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Reconstructing Evolutionary Histories of Calliphoridae (Diptera: Cyclorrhapha: Oestroidea) at Different Taxonomic Levels Using Phylogenomic Methods

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"The love for all living creatures is the most noble attribute of man."

— Charles Darwin

Lucilia sericata flies (Calliphoridae) at Toruń Główny railway station, Toruń, Poland, 2023. Photo by: Drashti R. Parmar.

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To Mummy & Papa...

Author's Declaration

I, Drashti R. Parmar, hereby confirm that the research presented in this thesis is my own original work undertaken at Department of Ecology and Biogeography, Faculty of Biological and Veterinary Sciences, Nicolaus Copernicus University in Toruń, between March 2021 to September 2025.

Collaborations and contributions by others are duly recognized, and my role in joint work is clearly stated in each article. Any previously published material is properly cited, with a complete list of publications provided in the bibliography. Details of collaborative efforts and co-authored publications are outlined at the beginning of relevant sections.

This thesis has been submitted as a *Thesis by Compilation*, in compliance with the Doctoral School of Academia Copernicana regulations. Four main chapters incorporate completely self-contained articles of which two of them have been published in a peer-reviewed journal, and the remaining two have been submitted in reputed international journals for publication.

I affirm that I have taken all reasonable steps to ensure the originality of this work. To the best of my knowledge, it does not violate any Polish education laws, infringe upon third-party intellectual property rights, or include unauthorized confidential material.

This thesis has not been submitted previously—in whole or in part—for a degree at this or any other institution.

The remaining thesis content was written independently by myself, incorporating feedback from my supervisors, Professor dr hab. Krzysztof Szpila and Dr. Nikolas P. Johnston.

Drashti R. Parmar

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"We're all mutants. What's more remarkable is how many of us appear to be normal."

—Dr. Walter Bishop, A fictional character, Fringe

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Finally, to my parents, who shaped the person I am today: every word in this thesis carries your sacrifices, love, and inspiration. This achievement is as much yours as it is mine.

Abstract

Blowflies (Diptera: Calliphoridae) represent a diverse, globally distributed family of calyptrate flies comprising ~2,075 species across 154 genera, representing 9% of calyptrate diversity and 1.3% of all described Diptera. Despite their ecological, medical, veterinary and economic significance as decomposers, pollinators, parasites, and vectors, blowfly diversity remains underestimated, and genomic resources are strikingly limited. Only ~1.6% of species have sequenced genomes, lagging behind other dipteran families, such as mosquitoes (Culicidae, 4%) and tsetse flies (Glossinidae, ~26%). These gaps limit our understanding of the evolutionary history of this group, explaining their ecological adaptations, and elucidating their roles in the transmission of disease-causing pathogens. This dissertation synthesizes multifaceted genomic, phylogenetic, and taxonomic studies aimed at filling significant knowledge gaps across the most important blowfly subfamilies. Leveraging large-scale mitochondrial datasets, it addresses systematic uncertainties and traces biogeographic and evolutionary patterns in key Calliphoridae lineages, with particular emphasis on the subfamilies Calliphorinae and Luciliinae.

A critical review of existing genomic resources for Calliphoridae in public databases reveals a clear tendency for researchers to focus on species of medical, veterinary and forensic importance. Other groups—often with diverse and highly interesting life strategies, including various forms of parasitism—are often overlooked. This highlights the urgent need to expand genomic data acquisition to a more representative range of taxa. Despite the availability of data for many key species of medical, veterinary, and forensic importance, the subfamilies Calliphorinae and Luciliinae continue to suffer from instability in the current taxonomy, uneven sampling of important genera, and a lack of genomic data even for common and widespread species. This limits the ability to obtain highly supported phylogenies and, consequently, infer the biogeography or trait evolution of these insects (**Parmar et al. 2025a**).

In response to the challenges outlined above, analyses of mitochondrial genomes were conducted for the broadest set of species from the Calliphorinae and Luciliinae subfamilies to date. One of the objectives was a comparative analysis of the mitochondrial genomes of 78 blowfly species, including 63 species, representing Calliphorinae subfamily. This analysis revealed extensive structural and functional

diversity in the mitogenome of individual species, such as gene rearrangements (e.g., in *Calliphora varifrons* Malloch) and base composition biases. The Phylogenomic reconstructions strongly support the monophyly of Calliphoridae. Crucially, it also resolves the long-debated relationships between the numerous blowfly subfamilies. An important result is the demonstrated paraphyly of some large Calliphorinae genera (e.g., *Calliphora* Robineau-Desvoidy and *Onesia* Robineau-Desvoidy), indicating an urgent need for taxonomic revisions (**Parmar et al. 2025b**).

The species-rich genus *Calliphora* Robineau-Desvoidy is of particular interest to researchers from various fields, including medical, veterinary, and forensic entomology. This research expanded the reference database of COI barcode sequences for Palearctic and Afrotropical species of this genus, adding 33 new sequences to the publicly available databases. Using this data, species delimitation for the taxa included in the analysis was tested. Using integrative taxonomy methods, two new *Calliphora* species were described from the highlands of Ethiopia (*Calliphora teraramma* and *Calliphora mesay*), a groundbreaking finding considering the previous presence of only one species of this genus in the Afrotropical Region. These results highlight the usefulness of molecular tools in revealing cryptic diversity and represent a significant contribution to the knowledge of blowfly diversity in the Afrotropical Region (**Parmar et al. 2025c**).

Leveraging the most comprehensive mitogenomic dataset of Luciliinae to date (32 species), the phylogenetic relationships of these flies were reconstructed. The resulting phylogenetic tree allowed us to trace the evolutionary history of the Luciliinae, determine the timing of divergence of the main evolutionary lineages, and identify ancestral bionomics. Analysis of the mitochondrial genome of Luciliinae, a group with remarkable trophic plasticity, revealed a surprising conservatism of the mitogenome. Results indicated a paraphyletic nature of the globally distributed genus *Lucilia* Robineau-Desvoidy. The resulting phylogenetic tree topology shows that all species of the other genera included in the analysis remain nested within this genus: *Blepharicnema* Macquart (1 species), *Hemipyrellia* Townsend (2 species), and *Hypopygiopsis* Townsend (4 species). This result challenges the current classification and indicates an urgent need for taxonomic revision. Dating the divergence of the major evolutionary lineages indicates that subfamily-level divergences occurred in the Oligocene–Miocene, and the subsequent radiation of Luciliinae was likely associated with global climatic changes and new ecological niches created by the expansion of

herbaceous vegetation and associated fauna. The Afrotropical region likely formed part of the ancestral range of the Luciliinae (~15–20 Ma), and subsequent diversification of its sister lineage gave rise to all the remaining Luciliinae in other geographic regions. Parasitism arose independently and repeatedly during Luciliinae evolution. Reconstructions of the ancestral life strategy indicate saprophagy as the ancestral habit, while obligate parasitism evolved convergently in the Late Pliocene (**Parmar et al. 2025d, submitted**).

Collectively, this dissertation significantly expands our understanding of blowfly phylogeny, systematics, evolution and biogeography. It also significantly fills the gap in our knowledge of the variability of the mitochondrial genome of Calliphoridae. The resulting phylogenetic reconstructions will provide a sound basis for future studies on trait evolution and necessary taxonomic revisions. The barcoding libraries enriched by this dissertation's contributions will significantly facilitate species identification in practice and research in medical, veterinary, and forensic entomology. The presented research also provides data for models tracking potential range shifts of parasitic species caused by climate change. This work highlights the importance of foundational taxonomic and genomic research on this globally important family of flies in addressing practical challenges related to biodiversity conservation and public health.

Streszczenie

Plujkowate (Diptera: Calliphoridae) są zróżnicowaną i kosmopolityczną rodziną owadów, obejmującą około 2075 gatunków zgrupowanych w 154 rodzajach. Stanowią one aż 9% różnorodności muchówek z grupy Calyptrata i 1,3% wszystkich znanych Diptera. Pomimo istotnego znaczenia ekologicznego, medycznego, weterynaryjnego i ekonomicznego jako organizmów rozkładających, zapylających, pasożytniczych i przenoszących choroby, różnorodność plujkowatych pozostaje niedoszacowana, czemu towarzyszy słabe poznanie zasobów genomowych. Genomy Calliphoridae sekwencjonowano jedynie dla 1,6% gatunków, co odstaje stanu poznania dla wielu rodzin muchówek, takich jak komarowate (Culicidae, 4%) czy muchy tse-tse (Glossinidae, ~26%). Te braki ograniczają możliwość zrozumienia historii ewolucyjnej grupy, wyjaśnienia występujących tu adaptacji ekologicznych czy roli tych muchówek w transmisji chorobotwórczych patogenów. Przedstawiona dysertacja jest syntezą wieloaspektowych badań genomowych, filogenetycznych i taksonomicznych, mających na celu uzupełnienie istotnych luk w wiedzy na temat najważniejszych podrodzin plujkowatych. Wykorzystując obszerne zestawy danych mitochondrialnych rozwiązano niejasności systematyczne, prześledzono wzorce biogeograficzne i ewolucyjne w kluczowych liniach rozwojowych Calliphoridae, ze szczególnym uwzględnieniem podrodzin Calliphorinae i Luciliinae.

Przegląd dostępnych zasobów genomowych dla Calliphoridae w publicznych bazach danych ujawnia wyraźne tendencje do skupiania się przez badaczy na gatunkach o znaczeniu medycznym, weterynaryjnym i sądowym. Pozostałe grupy – często o zróżnicowanych i niezwykle interesujących strategiach życiowych, obejmujących m.in. różne formy pasożytnictwa – pozostają często pomijane. Wskazuje to na pilną potrzebę rozszerzenia pozyskiwania danych genomowych na bardziej reprezentatywny zakres taksonów. Pomimo dostępności danych dla wielu kluczowych gatunków o znaczeniu medycznym, weterynaryjnym czy sądowym, podrodziny Calliphorinae i Luciliinae nadal dotyka niestabilność obowiązującej systematyki, nierównomierne próbkowanie ważnych rodzajów oraz brak danych genomowych nawet dla pospolitych i szeroko rozpowszechnionych gatunków. Ogranicza to możliwość otrzymywania wysokowspartych filogenez a co za tym idzie wnioskowania na temat biogeografii czy ewolucji cech tych owadów (**Parmar i in., 2025a**).

W odpowiedzi na przedstawione powyżej problemy, przeprowadzono analizy genomów mitochondrialnych dla najszerszego dotychczas zestawu gatunków z podrodzin Calliphorinae i Luciliinae. Jednym z zadań była analiza porównawcza genomów mitochondrialnych 78 gatunków plujkowatych, w tym 63 reprezentujących podrodzinę Calliphorinae. Analiza ta ujawniła znaczne zróżnicowanie strukturalne i funkcjonalne mitogenomu poszczególnych gatunków, takie jak rearanżacja genów (np. u *Calliphora varifrons* Malloch) oraz silnie zmienne proporcje składu zasad azotowych. Przeprowadzone rekonstrukcje filogenetyczne na bazie tego materiału zdecydowanie wspierają monofiletyczny charakter Calliphoridae. Przede wszystkim rozwiązują także dyskutowane od dłuższego czasu relacje pomiędzy licznymi podrodzinami plujkowatych. Ważnym rezultatem jest wykazana polifiletyczność niektórych dużych rodzajów Calliphorinae (np. *Calliphora* Robineau-Desvoidy i *Onesia* Robineau-Desvoidy), wskazująca na pilną potrzebę rewizji taksonomicznych (**Parmar i in. 2025b**).

Szczególne zainteresowanie badaczy z różnych dziedzin takich entomologia medyczna i weterynaryjna czy entomologia sądowa budzi bogaty w gatunki rodzaj *Calliphora* Robineau-Desvoidy. Dzięki prowadzonym badaniom rozszerzono bazę referencyjną sekwencji barkodowych COI dla palearktycznych i afrotropikalnych gatunków tego rodzaju, dodając do ogólnie dostępnym baz danych 33 nowe sekwencje. Wykorzystując te dane przetestowano delimitację granic gatunkowych dla włączonych do analizy taksonów. Stosując metody taksonomii integratywnej opisano dwa nowe gatunki *Calliphora* z obszarów górskich Etiopii (*Calliphora teraramma* oraz *Calliphora mesay*), co jest informacją sensacyjną wobec wcześniejszej obecności jedynie jednego gatunku z tego rodzaju w Regionie Afrotropikalnym. Wyniki te podkreślają przydatność narzędzi molekularnych w ujawnianiu kryptycznej różnorodności oraz stanowią istotny wkład w wiedzę o różnorodności plujkowatych w Regionie Afrotropikalnym (**Parmar i in., 2025c**).

Wykorzystując najbogatszy jak dotąd zestaw danych mitogenomowych dla podrodziny Luciliinae (32 gatunki), zrekonstruowano relacje filogenetyczne tych muchówek. Uzyskane drzewo filogenetyczne pozwoliło prześledzić historię ewolucyjną Luciliinae, określić czas dywergencji głównych linii ewolucyjnych i wskazać cechy ancestralne w bionomii. Analiza genomu mitochondrialnego Luciliinae, grupy o niezwykłej plastyczności troficznej, wykazała zaskakujący konserwatyzm mitogenomu. Uzyskano wyniki wskazujące na parafiletyczny charakter globalnie

rozprzestrzenionego rodzaju *Lucilia* Robineau-Desvoidy. Przy uzyskanej topologii drzewa filogenetycznego, wewnątrz tego rodzaju pozostają zagnieżdżone wszystkie gatunki pozostałych włączonych do analizy rodzajów: *Blepharicnema* Macquart (1 gat.), *Hemipyrellia* Townsend (2 gat.), *Hypopygiopsis* Townsend (4 gat.). Wynik ten podważa obecnie funkcjonującą klasyfikację i wskazuje na pilną potrzebę rewizji taksonomicznej. Datowanie dywergencji głównych linii ewolucyjnych wskazuje, że rozejścia na poziomie podrodzin miały miejsce w oligocenie–miocenie, a dalsza radiacja Luciliinae była prawdopodobnie związana ze zmianami klimatycznymi i nowymi niszami ekologicznymi powstałymi dzięki ekspansji roślinności trawiastej i związanej z tym środowiskiem teriofauny. Region afrotropikalny prawdopodobnie stanowił część zasięgu rodowego Luciliinae (~15–20 Ma), a późniejsza dywersyfikacja jego siostrzanej linii dała początek wszystkim pozostałym Luciliinae w innych regionach geograficznych. W czasie ewolucji Luciliinae wielokrotnie i niezależnie dochodziło do powstania pasożytnictwa. Rekonstrukcje ancestralnej strategii życiowej wskazują na saprofagia jako strategię ancestralną, natomiast pasożytnictwo obligatoryjne wyewoluowało konwergentnie w późnym pliocenie (**Parmar i in. 2025d, wysłane**).

Podsumowując, przedstawiona dysertacja znacząco poszerza wiedzę na temat filogenezy, systematyki, ewolucji i biogeografii plujkowatych. Z znaczący sposób wypełnia także lukę w wiedzy na temat zmienności genomu mitochondrialnego Calliphoridae. Uzyskane rekonstrukcje filogenetyczne będą stanowić dobrą podstawę dla przyszłych badań nad ewolucją cech i niezbędnych rewizji taksonomicznych. Wzbogacone poprzez wkład niniejszej dysertacji biblioteki barkodowe znacząco ułatwią identyfikację gatunkową materiału w praktyce i badaniach z zakresu entomologii medycznej, weterynaryjnej czy sądowej. Przedstawione badania dostarczają także danych dla modeli śledzących potencjalne zmiany zasięgów gatunków pasożytniczych, powodowane zmianami klimatu. Praca ta podkreśla znaczenie badań podstawowych tej globalnie istotnej rodziny muchówek dla zagadnień praktycznych związanych z ochroną bioróżnorodności czy zdrowiem publicznym.

Publications Included in the Doctoral Dissertation

Published and submitted articles are cited in bold throughout the thesis.

ARTICLE 1

Parmar, D. R.*, Johnston, N. P., Wallman, J. F., & Szpila, K. Blowfly genomics: current insights, knowledge gaps, and future perspectives. *Current Opinion in Insect Science.* 68, 101305 (2025a). https://doi.org/10.1016/j.cois.2024.101305.

Current Opinion in Insect Science, IF (4.8), Ministry of Science and Higher Education score: 100

ARTICLE 2

Parmar, D. R.*, Johnston, N. P., & Szpila, K. Large-scale comparative analysis of blowfly mitogenomes, with an emphasis on Calliphorinae (Diptera: Calliphoridae), provides evolutionary insights into mitochondrial DNA structure, gene rearrangements, and inter- and intra-subfamilial relationships among blowflies. *International Journal of Biological Macromolecules.* (2025b). 323(Pt 2):147063. https://doi.org/10.1016/j.ijbiomac.2025.147063.

International Journal of Biological Macromolecules, IF (8.5), Ministry of Science and Higher Education score: 100

ARTICLE 3

Parmar, D. R.*, Johnston, N. P., Dinka, M. D., & Szpila, K*. Species delimitation of the Afrotropical and Palaearctic *Calliphora* Robineau-Desvoidy and discovery of two new species in Afrotropics. *Medical and Veterinary Entomology*. (2025c, in press). https://doi.org/10.1111/mve.70011.

Medical and Veterinary Entomology, IF (1.9), Ministry of Science and Higher Education score: 140

ARTICLE 4

Parmar, D. R., Johnston, N. P., Pape, T., & Szpila, K*****. Mitogenomic phylogeny of the green bottles (Luciliinae: Calliphoridae: Oestroidea) reveals refined systematics, biogeographic patterns, mid-Miocene divergence and repeated evolution of parasitism.

Manuscript submitted to Molecular Biology and Evolution, IF (5.3), Ministry of Science and Higher Education score: 200

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INTRODUCTION

1.1 Overview of Blowfly Diversity and Evolutionary Significance

The family Calliphoridae (Diptera: Calyptratae), commonly known as blowflies, comprises ~2075 described species across 154 genera, representing approximately ~1.3% of known Diptera and ~9% of described calyptrates (Yan, Pape, et al. 2021; **Parmar et al. 2025a**). The family comprises of eight subfamilies named: Ameniinae, Bengaliinae, Calliphorinae, Chrysomyinae, Luciliinae, Phumosiinae, Rhiniinae, and Rhinophorinae, according to recent classification (Yan, Pape, et al. 2021). Despite accounting for a significant portion of calyptrate diversity, the true species richness is likely underestimated, particularly in tropical regions where taxonomic surveys and molecular sampling remains inadequate as with many Dipteran families (Yeates et al. 2007; **Parmar et al. 2025a**).

Blowflies occupy diverse ecological niches and exhibit a wide range of life histories, including necrophagy, pollination, facultative and obligate parasitism, and parasitoidism (Rognes K. 1991; Kutty et al. 2010). These adaptations make them significant players in decomposition, nutrient recycling, and host-parasite interactions (Rognes 1998). Some species, like *Lucilia sericata* Meigen, are medically relevant due to their role in myiasis and maggot debridement therapy (Davis et al. 2021), while others, such as *Cochliomyia hominivorax* Coquerel, are major veterinary pests (Scott et al. 2020; Tandonnet et al. 2023). The breadth of their biological functions has also made blowflies key organisms in forensic entomology, where precise species identification and larval growth rate informs post-mortem interval estimations (Wells and Stevens 2008).

Blowflies belong to the schizophoran grade of flies—an evolutionarily and behaviorally complex group that has contributed profoundly to biological research and public health studies (Rognes K. 1991; Yan, Pape, et al. 2021). Despite this applied relevance, many aspects of blowfly evolutionary biology remain poorly understood. Historically, the family has been underrepresented in genomic databases, particularly when compared to other dipteran families such as Drosophilidae and Culicidae (**Parmar et al. 2025a**). This underrepresentation hampers efforts to resolve species boundaries, trace evolutionary transitions like the emergence of parasitism, and understand the genetic basis of adaptation across varied environments.

1.2 Geographic Distribution and Diverse Life Histories of Calliphoridae

Calliphoridae are distributed across every major biogeographic realm, from Arctic tundra and alpine zones to tropical forests and deserts (Verves 2005), reflecting their remarkable ecological and physiological adaptability. Species richness is particularly high in the Holarctic and Australasian regions, yet the Afrotropics, Neotropics, and Southeast Asia also harbor a rich and largely unsampled diversity (Zumpt 1956; Rognes K. 1991; Rognes 1997; Williams and Villet 2006; Kelly et al. 2011; Tantawi et al. 2017; Lutz et al. 2018). Many blowfly taxa thrive in extreme or fragmented habitats, where their life history strategies are shaped by a combination of ecological opportunity and evolutionary plasticity.

Their larval feeding strategies span a continuum from saprophagy to obligate parasitism. Some species have specialized to feed on decomposing organic matter and play essential roles in ecosystem function through balancing nutrient recycling and carrion ecology (Stevens and Wall 1997; Fremdt et al. 2012). Others have independently evolved parasitic or parasitoid life strategies, targeting vertebrate and invertebrate hosts, ranging from facultative myiasis in open wounds (e.g., *Lucilia sericata*) to host-specific obligate endoparasitism of amphibians (e.g., *L. bufonivora* Moniez), reptiles, mammals or even snails (*Dyscritomyia* spp. Grimshaw) (Zumpt 1965; Tantawi and Whitworth 2014; Williams et al. 2016). This evolutionary plasticity appears to be influenced by both intrinsic biological traits (e.g., host specificity, reproductive strategies) and extrinsic environmental pressures (e.g., habitat fragmentation, climate variability, and human activity) (Hall and Wall 2005; **Parmar et al. 2025d, submitted**).

The subfamily Luciliinae, for instance, illustrates this ecological breadth, encompassing both decomposer and parasitic lineages (Stevens and Wall 1997). These transitions between feeding strategies, when studied in a phylogenetic and biogeographic context, can reveal how parasitism evolves, persists, and re-emerges in different lineages. Such behavioral and trophic diversity within Calliphoridae not only complicates their systematics but also means that blowflies are differentially impacted by ecological factors (Hall and Wall 2005). Understanding these dynamics are particularly relevant in a changing climate, where the distribution and behavior of blowflies may shift with profound ecological and medical consequences, making

blowflies excellent indicators for studying biotic responses to climate variability, habitat fragmentation, and anthropogenic disturbance (**Parmar et al. 2025a**).

Although blowflies are globally distributed, certain regions—particularly the Afrotropics—remain severely understudied in both morphological and genomic contexts. This lack of sampling is paradoxical, given that the Afrotropical region harbors a high diversity of endemic and cold-adapted blowfly species, especially in montane refugia such as the Ethiopian Highlands and the Great Rift Valley (**Parmar et al. 2025c**). Historically, only two species of *Calliphora* Robineau-Desvoidy—*C. croceipalpis* Jaennicke (indigenous) and *C. vicina* Robineau-Desvoidy (introduced)—were recognized in the region (Zumpt 1956; Williams and Villet 2006; Kelly et al. 2011; Lutz et al. 2018), highlighting the importance of targeted exploration and molecular surveys in these understudied biodiversity hotspots. The lack of genomic data from such regions limits our ability to conduct accurate species delimitation, resolve phylogenetic relationships, and assess biogeographic patterns, in turn constraining forensic and ecological applications. Addressing these knowledge gaps is critical for building a comprehensive evolutionary framework and ensuring that global analyses reflect true lineage diversity.

1.3 Systematics and Phylogenetic Challenges

The taxonomy and systematics of Calliphoridae have long been contentious with the group been repeatedly shown to be either para- or polyphyletic based on both molecular and morphological evidence (Rognes 1997; Kutty et al. 2010; Narayanan Kutty et al. 2019; Buenaventura et al. 2021; Yan, Pape, et al. 2021). Morphological convergence, limited taxon sampling, and reliance on homoplastic characters have complicated efforts to resolve higher-level phylogenies. Morphological data alone have often yielded contradictory topologies and consequently failed to resolve deep evolutionary relationships (Kutty et al. 2010; Marinho et al. 2012), prompting the adoption of molecular phylogenetics to clarify subfamily and genus-level boundaries.

In particular, progress in blowfly systematics and evolutionary biology has been hindered by taxonomic ambiguities, sparse genomic sampling, and methodological inconsistencies across studies (**Parmar et al. 2025a**). However, the taxonomy of Calliphoridae has undergone significant revision in recent decades, fueled by advances in molecular phylogenetics. Recent molecular evidence has prompted major revisions

in this lineage: formerly separate subfamilies such as Aphyssurinae, Melanomyinae, and Toxotarsinae have been merged into Calliphorinae, while Polleniidae and Mesembrinellidae have been elevated to family rank (Singh and Wells 2013; Cerretti et al. 2019; Buenaventura et al. 2021; Yan, Pape, et al. 2021). Rhiniinae and Rhinophorinae, previously treated as separate families or ambiguous subgroups, are also now recognized as valid subfamilies within Calliphoridae (Buenaventura et al. 2021; Yan, Pape, et al. 2021; Cerretti et al. 2024; **Parmar et al. 2025b**). Despite these advances, several subfamilial relationships remain unresolved, often hindered by poor representation of non-model taxa and endemic lineages. For instance, relationships among lineages lacking genomic representation, such as Phumosiinae, Bengaliinae, and Ameniinae are still ambiguous (**Parmar et al. 2025a**). Discrepancies persist between datasets, particularly regarding the often contested monophyly of Calliphoridae and its subfamilies.

The genera *Calliphora* and *Lucilia* Robineau-Desvoidy further exemplify these challenges. While both are widely used in forensic and veterinary contexts, their genus and species-level phylogenies remain unclear (**Parmar et al. 2025b; Parmar et al. 2025d, submitted**). For instance, key relationships within the Calliphorinae remain contentious, such as the unstable placement of genera *Cyanus* Hall, *Cynomya* Robineau-Desvoidy and *Cynomyiomima* Rohdendorf inside *Calliphora* lineage. Luciliinae, including genera such as *Blepharicnema* Macquart, *Dyscritomyia*, *Hemipyrellia* Townsend, *Hypopygiopsis* Townsend and *Lucilia* presents a similarly complex case. This subfamily exhibits diverse larval feeding strategies, including facultative and obligate parasitism and carrion-breeding, however, its evolutionary relationships, particularly the monophyly of *Lucilia*, remain poorly resolved (**Parmar et al. 2025d, submitted**). Compounding these problems is the heavy reliance on medically and economically significant species for molecular data—namely *L. cuprina* Wiedemann and *L. sericata*—with many endemic or ecologically unique taxa underrepresented (**Parmar et al. 2025a**). Additionally, mitogenomic data and COI barcodes have also often yielded conflicting topologies, likely due to incomplete sampling and low genetic divergence among morphologically similar taxa (**Parmar et al. 2025c**).

These taxonomic challenges illustrate the urgent need for integrative and phylogenomically robust datasets encompassing broader sampling across diverse

habitats to clarify lineage boundaries, infer trait evolution, and support systematic revisions.

1.4 Advances in Genomics and their Applications in Blowfly Research

Advances in sequencing technology and analytical frameworks have transformed the field of invertebrate systematics. High-throughput short-read platforms (e.g., Illumina) have enabled the rapid assembly of mitochondrial and draft nuclear genomes, while long-read technologies (e.g., PacBio and Oxford Nanopore) have facilitated chromosome-level genome assemblies (Hotaling et al. 2021). These tools have provided valuable insights into genome architecture, gene content, and structural variations within several insect groups (Cameron 2014).

Phylogenomics has benefited greatly from targeted sequencing methods such as Anchored Hybrid Enrichment (AHE), Ultraconserved Elements (UCEs), and transcriptomics (Buenaventura et al. 2020; Buenaventura et al. 2021; Yan, Pape, et al. 2021; Johnston et al. 2024). These approaches have helped resolve complex phylogenies across Diptera. Within the Dipteran superfamily Oestroidea, such techniques have clarified relationships among families, including Calliphoridae and have led to the redefinition of family boundaries, and revealed patterns of genomic conservation and divergence. For example, the raising of Mesembrinellidae and Polleniinae to family status is strongly supported by nuclear phylogenomic data (Buenaventura et al. 2021; Yan, Pape, et al. 2021; Cerretti et al. 2024). However, these methods vary in their suitability depending on DNA quality, genome complexity, and taxonomic scale. Nonetheless, many calliphorid subfamilies (e.g., Ameniinae, Bengaliinae, Phumosiinae) remain without any genomic representation, limiting our ability to infer evolutionary relationships at a family-wide scale (**Parmar et al. 2025a**).

Genomic efforts remain disproportionately focused on species of medical, veterinary, and forensic relevance, leaving ecologically significant or geographically isolated taxa underrepresented. For instance, fewer than 1% of known blowfly species have genome assemblies publicly available (**Parmar et al. 2025a**). Even within subfamilies like Luciliinae and Calliphorinae, genomic coverage is skewed toward a few genera, predominantly *Lucilia* and *Calliphora*. In particular, species like *C. vicina*, *C. vomitoria* Linnaeus, *L. cuprina* and *L. sericata* have received disproportionately greater attention in genomic studies, largely due to their forensic and veterinary

importance. The uneven taxonomic and geographic coverage of genomic data restricts the scope of comparative studies and impairs our ability to interpret functional and evolutionary processes across the family (**Parmar et al. 2025b; Parmar et al. 2025d, submitted**).

Expanding taxon sampling and geographical coverage is imperative for reconstructing evolutionary trajectories, uncovering adaptive mechanisms, and resolving phylogenetic relationships. Importantly, genome-wide data also enable investigation into traits such as host specificity, immune evasion, dispersal potential, and insecticide resistance—critical for managing species that affect agriculture, public health, and biodiversity.

1.5 Mitochondrial Genomics in Blowfly Systematics

Mitochondrial genomes (mitogenomes) are widely used in insect systematics due to their high copy number, conserved structure, maternal inheritance, and relatively rapid mutation rates (Cameron 2014). Besides resolving deeper phylogenetic relationships, mitogenomes, particularly in Diptera have provided valuable insights into nucleotide composition, codon usage, substitution rates, compositional biases, and gene rearrangements (Li et al. 2020; Yan, Xu, et al. 2021; Song et al. 2023; Pei et al. 2024). Mitogenomes are also effective tools for species identification, particularly in cases of cryptic diversity.

Nonetheless, the available mitogenomic data for Calliphoridae is sparse and unevenly distributed across subfamilies. Of the >2,000 described species, fewer than 3% are represented by complete or near-complete mitogenomes in public databases, with a strong bias toward *Calliphora*, *Lucilia*, and other forensically relevant genera (**Parmar et al. 2025a**). For instance, within Calliphorinae, only 25 mitogenomes representing 16 species are available, and most belong to a single genus i.e., *Calliphora* (**Parmar et al. 2025b**). This imbalance limits our ability to rigorously test hypotheses on trait evolution, divergence times, and lineage relationships—particularly in subfamilies like Luciliinae and Calliphorinae, which are characterized by complex ecological transitions.

1.6 Species Delimitation and Cryptic Diversity

Integrative taxonomic approaches—combining morphology, DNA barcoding, and phylogenetics—have emerged as a powerful method for resolving species boundaries, particularly in cryptic or poorly studied taxa, leading to the discovery of hundreds of insect species, particularly in underexplored regions (Pont 1980; Johnston et al. 2020; Antil et al. 2023). In blowflies, DNA barcoding using the COI gene has proven useful for identifying closely related species and supporting forensic and ecological applications (Lutz et al. 2018; Antil et al. 2023; Johnston et al. 2025).

Despite the power of DNA barcoding for species delimitation, blowflies remain underrepresented in major public databases. For example, only ~16.5% of described Calliphoridae species are covered in the Barcode of Life Data Systems (BOLD), with nearly a quarter of sequences lacking formal taxonomic assignment (**Parmar et al. 2025a**). This underrepresentation is particularly pronounced in biodiversity-rich regions like the Afrotropics. For instance, the genus *Calliphora*, though well studied in temperate zones, remains poorly sampled in tropical montane habitats (**Parmar et al. 2025c**). This taxonomic shortfall hinders biodiversity assessments, species diagnostics, and ecological applications.

Efforts to build regional and global barcode libraries with well-annotated sequences from diverse habitats are thus critical. Targeted sequencing of unsampled or underrepresented lineages will not only clarify species boundaries but also support forensic, medical, and conservation-oriented research.

1.7 Genome Skimming as a Tool for Phylogenomics

Genome skimming—a low-coverage whole-genome sequencing approach—has emerged as a cost-effective and scalable method for recovering mitogenomes and other high-copy genomic elements from both fresh and archival specimens (Trevisan et al. 2019; Johnston et al. 2023). Especially effective for degraded DNA, this approach has made it possible to include museum collections and rare taxa in large-scale phylogenetic analyses, bypassing the need for extensive optimization or high-molecular-weight DNA (Lemmon and Lemmon 2013; Johnston et al. 2023).

In blowfly research, genome skimming offers a scalable solution for expanding taxonomic and geographic coverage across Calliphorinae and Luciliinae (**Parmar et al. 2025b; Parmar et al. 2025d, submitted**). By overcoming traditional barriers of

specimen preservation and sequencing cost, it may significantly support phylogenetic reconstruction, species delimitation, and trait mapping across underrepresented taxa. Furthermore, the approach can potentially lay the groundwork for integrating nuclear data—e.g., ribosomal genes and repetitive elements—into broader phylogenomic analyses. Genome skimming thus plays a pivotal role in democratizing access to genomic resources, particularly for researchers working in biodiversity-rich but resource-limited regions.

1.8 Research Goals and Hypotheses

The family Calliphoridae, with its ecological diversity, complex evolutionary history, and applied significance, serves as an excellent model for studying evolutionary dynamics, including rapid radiations and trait evolution in Diptera. The present work seeks to address critical gaps in our understanding of calliphorid systematics and phylogeny through an integrative approach that leverages both morphological and genomic data.

Specifically, this study aims to:

1. Identify and address critical genomic gaps in Calliphoridae by promoting comprehensive, subfamily-wide genome sequencing to advance species identification, systematics, and evolutionary research (**Parmar et al. 2025a**).
2. Resolve subfamily and genus-level phylogenetic relationships within Calliphoridae, with emphasis on Calliphorinae and Luciliinae through comprehensive taxon sampling, using efficient phylogenomic strategies, e.g., genome skimming, mitochondrial phylogenomics, and comparative mitogenomic analyses (**Parmar et al. 2025b; Parmar et al. 2025d, submitted**).
3. Clarify species boundaries and discover cryptic diversity through integrative taxonomy, with a focus on underexplored regions like the Afrotropical highlands (**Parmar et al. 2025c**).
4. Reconstruct the evolutionary history of parasitic traits and infer transitions between saprophagy and parasitism in a biogeographic context, particularly in Luciliinae (**Parmar et al. 2025d, submitted**).

5. Establish a dated phylogeny for Calliphoridae, providing a foundation for evolutionary, ecological, forensic, and applied entomological research (**Parmar et al. 2025d, submitted**).

Taken together, these goals aim to fill major gaps in blowfly taxonomy and mitogenome evolution, expand critical molecular resources, and offer insights into evolutionary trajectories within this rapidly radiating insect group. Our work establishes a robust framework for future ecological, forensic, and applied entomological studies of ecologically and economically significant blowfly lineage.

MATERIALS & METHODS

2.1 Specimen Collection across Calliphoridae

Adult blowfly specimens were freshly collected during fieldwork across multiple continents using entomological nets baited with decomposing vertebrate remains. All the specimens were either preserved in ethanol or maintained as pinned vouchers. In addition to field-collected material, several specimens were obtained from preserved museum collections to broaden taxonomic and geographic representation. A summary of all specimens, including collection details and accession information, is provided in Table T1 and Table S1 (**Parmar et al. 2025b, 2025c and Parmar et al. 2025d, submitted**).

Where possible, multiple individuals of the same species were sampled from geographically distinct regions to assess intraspecific divergence and infer potential dispersal patterns between isolated populations. Taxonomic identification and classification used in this study follow Rognes K. (1991) and Yan, Pape et al. (2021).

In total, 129 specimens were used for generating mitochondrial genomes assemblies, representing 107 blowfly species across 29 genera, encompassing all eight recognized calliphorid subfamilies (Table T1). To enhance phylogenetic coverage, additional 45 reference mitogenomes from GenBank (NCBI) were included, making a final mitochondrial dataset of 115 blowfly species across 36 genera. For most phylogenetic analyses, three additional species representing Mesembrinellidae (*Mesembrinella benoisti* Séguy, *Mesembrinella* sp.) and monotypic Ulurumyiidae (*Ulurumyia macalpinei* Michelsen & Pape) were selected as outgroups (Table T1).

Depending on the analysis, different mitogenome datasets were curated and used, these methods included: genome characterization, phylogenetic inference, species delimitation, barcode library generation, divergence time estimation, and ancestral state reconstruction. Full details of dataset construction and their applications are provided in **Parmar et al.** (**2025b, 2025c and Parmar et al. 2025d, submitted**).

2.2 Morphological Identification and Digital Documentation

Specimens were identified to the genus level using standard taxonomic keys provided by Zumpt (1956, 1965) and Rognes K. (1991, 1998). Terminology for adult morphology follows Rognes K. (1991), with updates adopted from Merz and Haenni (2000) and Zatwarnicki T. (1996).

Body length measurements were recorded for all type specimens, while detailed morphological measurements—particularly of diagnostic traits—were taken from holotypes. Male terminalia were dissected and preserved as morphological vouchers for future reference. All voucher specimens are housed at the Department of Ecology and Biogeography, Nicolaus Copernicus University in Toruń, Toruń, Poland.

External morphology was digitally documented at Nicolaus Copernicus University in Toruń using a Leica M205 C stereomicroscope equipped with a high-resolution Leica DFC495 digital camera. Image stacks (30–35 layers per specimen) were processed using Leica Application Suite 4.4.0 software to generate composite, high-resolution images with extended depth of field.

2.3 DNA extraction, Library Preparation and Illumina Sequencing

Total genomic DNA was extracted from 1–2 legs of pinned specimens or thoracic muscle tissue from ethanol-preserved samples using a DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA). DNA was eluted twice in 50 µl TE buffer (total 100 µl) and stored at –80 °C for downstream applications. DNA concentration was quantified using a Qubit 3.0 fluorometer with the dsDNA High Sensitivity Assay Kit (Life Technologies, Carlsbad, CA, USA), and DNA integrity was assessed by 0.5% agarose gel electrophoresis stained with GelRed (Biotium, Darmstadt, Germany).

Genome skimming was performed to construct genomic libraries, following Trevisan et al. (2019) and Johnston et al. (2023), with minor modifications outlined below. Input genomic DNA was mechanically sheared using a Covaris M220 ultrasonicator (Covaris, Brighton, UK), and two pooled libraries were prepared using the NEBNext Ultra II DNA Library Prep Kit (New England BioLabs, Ipswich, MA, USA) with an average insert size of ~200 base pairs (bps). Libraries were indexed using NEBNext Multiplex Oligos for Illumina and assessed for quality and fragment size distribution on an Agilent 2100 Bioanalyzer with a high-sensitivity DNA chip (Agilent Technologies, Santa Clara, CA, USA). Pippin Prep (Sage Science, Beverly, MA, USA) was used for size selection of 170–400 bp and to remove library fragments containing only adapters.

Sequencing was conducted on an Illumina NovaSeq 6000 platform (paired-end, 100 bp reads) at Macrogen Europe (Amsterdam, The Netherlands). Post-sequencing

quality filtering was performed using Trimmomatic (Bolger et al. 2014), yielding an average of ~1 Gb of clean data per library for downstream bioinformatic analyses.

2.4 Mitochondrial Genome Assembly and Annotation

De novo assemblies were performed using MitoZ v3.6 (Meng et al. 2019) with the MegaHit v1.2.9 assembler (Li et al. 2016), successfully recovering over 80% of genomes. For the remaining incomplete assemblies, three additional pipelines were employed via MitoFinder v1.4.2 (Allio et al. 2020), as implemented recently with calyptrates (Johnston et al. 2023): (1) metaSPADES v4.2.0 (Nurk et al. 2017), (2) MegaHit v1.2.9 (Li et al. 2016), and (3) IDBA-UD v1.1.3 (Peng et al. 2012), using *Lucilia cuprina* (GenBank: JX913744, Nelson et al. 2012) as a reference. Combined, these methods yielded complete or near-complete mitogenomes for all specimens. Assembly quality and completeness were validated against published mitogenomes of closely related species in NCBI.

Annotation of protein-coding genes (PCGs), rRNAs, and tRNAs was conducted using a combination of tools: MiTFi v0.1 (Jühling et al. 2012) in MitoZ and ARWEN v1.2.3 (Laslett and Canbäck 2008) in MitoFinder for tRNAs, with tRNA secondary structures predicted using tRNAscan-SE 2.0 (https://trna.ucsc.edu/tRNAscan-SE/) (Chan and Lowe 2019). PCGs and rRNAs were further confirmed using tblastn (Gertz et al. 2006) and MITOS2 (Bernt et al. 2013), respectively. The A+T-rich control region was identified using Tandem Repeats Finder v4.10.0 (Benson 1999). Partial or missing gene regions were annotated via MITOS2 on the Galaxy platform (https://usegalaxy.eu/) (Afgan et al. 2018). Additionally, MITOS2 was used to annotate eight Calliphoridae mitogenomes retrieved from NCBI. Mitogenome maps were visualized using the CGView server (http://stothard.afns.ualberta.ca/cgview_server/) (Stothard and Wishart 2005). All newly assembled sequences were submitted to GenBank (Table T1).

Full-length COI sequences (1536 bp) used for barcode reference library construction and species delimitation (**Parmar et al. 2025c**) were extracted from annotated mitogenomes using MitoZ and verified against NCBI references for length and accuracy. These COI sequences are deposited in the BOLD Systems database (Ratnasingham and Hebert 2007) under the dataset DS-KEIBNCU, Barcode Library for

Afrotropical and Palaearctic *Calliphora* (doi: dx.doi.org/10.5883/DS-KEIBNCU) (**Parmar and Szpila 2025**) (Table T1).

2.5 Mitogenome Characterization and Comparative Analyses

This section describes analyses conducted for mitochondrial characterization (**Parmar et al. 2025b; Parmar et al. 2025d, submitted**) and comparative mitogenomics (**Parmar et al. 2025b**) within Calliphoridae, with a focus on Calliphorinae and Luciliinae. Entire mitogenomes, as well as individual alignments for protein-coding genes (PCGs), tRNAs, and rRNAs, were performed using the L-INS-i algorithm in MAFFT v7.490, implemented in Geneious Prime v2024.0.2. Nucleotide composition and relative synonymous codon usage (RSCU) for each PCG were calculated in MEGA v11.0 (Tamura et al. 2021). Strand asymmetry (AT-skew and GC-skew) was manually computed for all species using the formula, AT-skew = [A – T] / [A + T] and GC-skew = [G – C] / [G + C] (Perna and Kocher 1995).

Substitution saturation in PCGs was assessed by plotting transitions and transversions against TN84 distances using DAMBE v7.0 (Xia 2018). Iss and Iss.c values (Xia et al. 2003; Xia and Lemey 2009) were also calculated to evaluate the reliability of phylogenetic inferences (**Parmar et al. 2025b:** Table S3). Non-synonymous (Ka) and synonymous (Ks) substitution rates, as well as the Ka/Ks ratio, were estimated in DnaSP v6.12.03 (Librado and Rozas 2009) to evaluate evolutionary pressures on PCGs.

To assess subfamily-level differences in base composition and strand asymmetry, Kruskal–Wallis tests (Kruskal and Wallis 1952) followed by Dunn's post hoc comparisons (Dunn 1964) (adjusted with the Benjamini–Hochberg correction; Benjamini and Hochberg 1995) were conducted in R v4.4.1. Comparative analyses of genome architecture—including gene rearrangements, duplications, and losses—were carried out manually in Geneious Prime v2024.0.2, while repetitive elements potentially associated with structural variation were identified using REPuter (Kurtz 2001).

2.6 DNA Barcoding and Species Delimitation Analysis

This section describes the methodology used in **Parmar et al. 2025c**. To test the validity of morphologically defined species hypotheses and evaluate the effectiveness

of DNA barcodes in delimiting *Calliphora* and *Cynomya* species from the Palaearctic and Afrotropical regions, we applied two complementary species delimitation methods: Automatic Barcode Gap Discovery (ABGD; Puillandre et al. 2012) and Assemble Species by Automatic Partitioning (ASAP; Puillandre et al. 2021). Both analyses were conducted using the SPART Explorer web platform (https://spartexplorer.mnhn.fr/), which integrates species delimitation and comparison through a three-step workflow: (1) application of ABGD and ASAP, (2) visualization of species partitions using interactive segmented bar charts, and (3) statistical evaluation of partition congruence via the LIMES module (Ducasse et al. 2020).

For ABGD, we set the prior intraspecific divergence (P) from 0.001 to 0.1 in 20 steps, with X-values ranging from 1.0 to 1.2. ASAP was run with a group split threshold of 0.01, using the simple distance method (Collins et al. 2012), which has proven effective for closely related species (Srivathsan and Meier 2012; Johnston et al. 2023). Final partitions were selected based on the lowest ASAP score and compared with ABGD results. Congruence between molecular and morphological species hypotheses was further evaluated using the LIMES module.

To complement the SPART results, pairwise genetic distances were calculated using the Kimura 2-parameter (K80) model in R via the *ape::dist.dna()* function, treating gaps by pairwise deletion. Intra- and interspecific distances were also computed using the adegenet package (v2.1.10; Jombart 2008) in R v4.3.2 within RStudio v2025.05.0+496 (Posit, Boston, USA).

2.7 Phylogenetic Analyses

The 13 mitochondrial protein-coding genes (PCGs) were individually aligned using MAFFT v7.490 (L-INS-i algorithm) (Katoh and Standley 2013) and concatenated into a supermatrix using SequenceMatrix v1.8.2 (Vaidya et al. 2011). Poorly aligned regions and outliers were manually removed. The concatenated dataset was partitioned by gene and codon position, and the best-fit substitution models were determined using ModelFinder (Kalyaanamoorthy et al. 2017) based on the Akaike (AIC) or Bayesian (BIC) information criteria, depending on the study (**Parmar et al. 2025b:** Table S4; **Parmar et al. 2025d, submitted:** Table S2).

Maximum Likelihood (ML) phylogenies were inferred using IQ-TREE v2.1.4-beta (Minh et al. 2020) with node and branch support evaluated using ultrafast bootstrap

(Hoang et al. 2018) and SH-like approximate likelihood ratio tests (Guindon et al. 2010), each with 10,000 replicates. Bayesian Inference (BI) analyses were performed using ExaBayes v1.5 (Aberer et al. 2014) with eight or sixteen (depending on dataset size) independent MCMC chains (run for 1.5 million generations, sampling every 1,000 generations, 25% burn-in). All priors were kept as default values. Convergence of parameter estimates was confirmed using Effective Sample Size (ESS ≥ 200) and Potential Scale Reduction Factor (PSRF < 1.1), assessed via the 'postProcParam' tool, Topological convergence among chains was evaluated using the Average Standard Deviation of Split Frequencies (ASDSF < 0.05). An extended majority-rule consensus tree was then constructed from the post-burn-in tress using the 'consense' tool.

To account for potential compositional bias, additional ML and BI analyses were performed on two modified datasets: (1) third codon position removed (ML12/BI12) and (2) RY-coded (MLRY/BIRY). Trees from all datasets were compared to assess topological stability and data reliability.

To test alternative hypotheses regarding the monophyly of *Calliphora* and *Lucilia*, constrained ML trees were generated enforcing monophyly under the same partitioning and model conditions. These were statistically compared to the best ML tree using Approximately Unbiased (AU) (Shimodaira 2002), Shimodaira–Hasegawa (SH) (Shimodaira and Hasegawa 1999), and Kishino–Hasegawa (KH) tests (Kishino and Hasegawa 1989), implemented in IQ-TREE with 10,000 Resampling Estimated Log-Likelihoods (RELL) bootstrap replicates (-au option) (Kishino et al. 1990).

For species delimitation analyses, 177 COI barcode sequences (658 bp) representing *Calliphora* and *Cynomya* species from the Palaearctic and Afrotropical regions were assembled by combining newly generated data with sequences from BOLD and NCBI (**Parmar et al. 2025c:** Table S2). Sequences were curated for quality, completeness, and taxonomic consistency, with pseudogenes and Nuclear Mitochondrial DNA Segments (NUMTs) removed in Geneious Prime v2024.0.2. Synonymies with *Aldrichina* Townsend and *Triceratopyga* Rohdendorf were resolved following recent taxonomic updates (Rognes K. 1991; Park et al. 2009; Park et al. 2013). Barcode sequences were aligned using MAFFT v7.490 and then analyzed in IQ-TREE under the ML criteria with the TIM2+F+I+G4 substitution model, selected as best-fit model in by ModelFinder. Node and branch support was evaluated using 10,000 ultrafast bootstrap replicates and SH-like aLRTs. *Pollenia rudis* Fabricius was used to root the tree.

All phylogenies were visualized in FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) and edited using Inkscape 1.3.2 (available at https://inkscape.org).

2.8 Divergence Time Estimation and Biogeographic Structuring

This section describes the methodology used in **Parmar et al. (2025d, submitted)**. To estimate the evolutionary history of Calliphoridae, especially within green bottles (Luciliinae) across their biogeography, we estimated divergence times using a Bayesian MCMC framework in BEAST v2.7.7 (Bouckaert et al. 2019). We applied an uncorrelated log-normal relaxed clock (Drummond et al. 2006) together with a Yule speciation prior. Three calibration points, based on Junqueira et al. (2016), were used to constrain key nodes: the origins of Oestroidea, Calliphoridae, and the Calliphorinae + Luciliinae clade (**Parmar et al. 2025d, submitted:** Table S3). Two independent MCMC runs were conducted for 100 million generations, sampling every 1,000 generations and discarding 25% as burn-in. Convergence and ESS values (≥ 200) were assessed using Tracer v1.7.2 (Rambaut et al. 2018). A maximum clade credibility tree with mean node ages was generated using TreeAnnotator v2.7.7 (Drummond and Rambaut 2007; Berling et al. 2025).

2.9 Ancestral State Reconstruction of Larval Feeding Strategies

This section describes the methodology used in **Parmar et al. (2025d, submitted)**. To investigate the evolutionary history and directional transitions of larval feeding strategies in, especially in Luciliinae, we conducted ancestral state reconstruction (ASR) using a maximum likelihood (ML) framework based on a time-calibrated ultrametric tree. Larval feeding modes for each terminal taxon were categorized into one of six discrete states: coprophagy, facultative parasitism, obligatory parasitism, predation, saprophagy and a mixed state (facultative parasitism + predation), based on published literature (**Parmar et al. 2025d, submitted:** Table S4). Species with unknown biology were coded as "NA" and treated as having equal probabilities across all six states. Facultatively parasitic taxa, including *Lucilia cuprina* and *L. sericata*, were classified accordingly due to their documented role in primary or secondary myiasis (Cardoso et al. 2025).

Marginal ancestral state estimation was performed using the *fitMk()* and *ace()* functions from the R packages phytools v2.0 (Revell 2024) and ape v5.8-1 (Paradis and Schliep 2019), assuming equal transition rates among states. To incorporate uncertainty and estimate transition frequencies, stochastic character mapping (SCM) was conducted with *make.simmap()* under 1,000 MCMC replicates.

We tested three transition rate models—equal rates (ER), symmetrical (SYM), and all rates different (ARD)—using AICc values and model weights for comparison (Harmon 2019). To visualize trait evolution, model-averaged marginal reconstructions were generated using *ancr()*, with feeding states color-coded and displayed as pie charts on nodes using *plotTree()* in phytools.

RESULTS

3.1 Mitochondrial Genome Architecture

3.1.1 Mitogenome Structure and Composition

We assembled 129 calliphorid mitogenomes, representing 107 blowfly species across 29 genera, encompassing all eight recognized calliphorid subfamilies (Table T1). The mitogenomes were 14–19 kb in size, comprising 13 PCGs, 22 tRNAs, 2 rRNAs, and an AT-rich control region (**Parmar et al. 2025b:** Fig. 1). Gene order was highly conserved across most mitogenomes. Calliphoridae mitogenomes, including PCGs were strongly A+T biased, with variable A+T content across genera, ranging from 75%–82%. All mitogenomes showed positive AT skew and negative GC skew, inverted in PCG regions (**Parmar et al. 2025b:** Table 2; **Parmar et al. 2025d, submitted:** Table S5).

Statistical tests confirmed significant inter-subfamily differences in base composition and strand asymmetries (Kruskal–Wallis, $p < 0.01$; Dunn's test, $p < 0.05$) (**Parmar et al. 2025b:** Table 2; Table S5; Table S6). Calliphorinae and Luciliinae had lower skew and compositional bias compared to more derived or ecologically distinct groups like Rhinophorinae.

3.1.2 Codon Usage Patterns

Most PCGs began with standard ATN start codons, except for COI, ND1, and ND5, which used non-canonical alternatives (e.g., TCG, TTG, GTG), consistent with other Dipteran mitogenomes (Stevens et al. 2008; Nelson et al. 2012; Shang et al. 2022). Truncated stop codons (T/TA) were detected in several PCGs, consistent with post-transcriptional polyadenylation (Nelson et al. 2012).

Relative Synonymous Codon Usage (RSCU) analyses showed a strong preference for A/U-ending codons, especially for Leu (UUA), Ser (UCU), and Gly (GGA) (**Parmar et al. 2025b:** Fig. 2, Fig. S2). These biases correlated with overall AT richness. Frequently used codons also corresponded to tRNAs with complementary anticodons, suggesting selection for translational efficiency (**Parmar et al. 2025b:** Table S7).

3.1.3 Evolutionary Rate Variation

Synonymous substitution rates (Ks) across PCGs were relatively consistent across subfamilies whereas non-synonymous rates (Ka) varied widely (**Parmar et al. 2025b:**

Fig. 3A & 3B). Ameniinae and Rhinophorinae exhibited elevated Ka and Ka/Ks (ω) ratios (**Parmar et al. 2025b:** Fig. 3B & 3C), suggesting lineage-specific relaxed purifying selection or potential adaptive evolution in distinct ecological niches. In contrast, Calliphorinae, Chrysomyinae and Luciliinae showed low ω values (0.03–0.06), indicative of strong purifying selection and functional conservation. Gene-specific analyses showed ATP8, ND2, and ND6 had the highest ω values, while COI was the most conserved (ω = 0.04), reaffirming its utility as a barcode marker (Oliveira et al. 2008).

3.1.4 Gene Rearrangement Events

Structural rearrangements within mitogenomes were rare across Calliphoridae but notable. All major rearrangements occurred within Calliphorinae. *Calliphora varifrons* Malloch (PQ594010) showed a rare inverse transposition and fragmentation of the rrnL gene, supported by repeat elements consistent with the Tandem Duplication–Random Loss (TDRL) model (**Parmar et al. 2025b:** Fig. 4). Additional rearrangements included strand switches (e.g., CYTB in *Polleniopsis mongolica* Séguy; MN131052) and gene fragmentation (e.g., ATP6 in *Calliphora uralensis* Villeneuve; PQ594004).

Approximately 75% of detected rearrangements involved tRNAs, often species- or population-specific. *Bellardia bayeri* Jacentkowský (OX493271) had a novel trnI triplication (including a divergent copy), while duplication patterns varied across geographically distinct populations of *Calliphora subalpina* Ringdahl, *Bellardia pandia* Walker and others (**Parmar et al. 2025b:** Table 3). No significant correlation was found between tRNA duplication and genome composition, selection, or strand asymmetry.

3.2 Phylogenetic Relationships Across Calliphoridae

The results described under this heading correspond to the phylogenetic trees presented in **Parmar et al. 2025b:** Fig. 5; Fig. S3–S7, **Parmar et al. 2025c:** Fig. S1 and, **Parmar et al. 2025d, submitted:** Fig. 1–2; Fig. S2–S3.

3.2.1 Phylogenetic Framework for Calliphoridae

Phylogenetic analyses using mitogenomic data from 115 calliphorid species representing all eight recognized subfamilies yielded well-resolved and largely congruent topologies across Maximum Likelihood (ML) and Bayesian Inference (BI) frameworks. Calliphoridae was consistently recovered as monophyletic, with Mesembrinellidae and Ulurumyiidae forming a sister group (posterior probability [PP] = 1.0; SH-aLRT = 97.6; ultrafast bootstrap [UFB] = 91). All subfamilies except Phumosiinae (represented by a single taxon) were recovered as monophyletic.

Chrysomyinae formed a robust sister group to the combined Luciliinae + Calliphorinae clade (PP = 1.0; SH-aLRT = 94.8; UFB = 80). Rhiniinae and Rhinophorinae were confirmed as valid calliphorid subfamilies. The placement of *Eurychaeta palpalis* Robineau-Desvoidy varied across datasets, indicating phylogenetic instability. In different analyses, it formed a clade with Ameniinae (PP = 0.61), grouped with Rhinophorinae (SH-aLRT = 58.8; UFB = 82), or appeared as an independent lineage with strong support (PP = 1.0; SH-aLRT = 92.7; UFB = 93). These results suggest that the position of *E. palpalis* is unstable and may reflect either limited phylogenetic signal or conflicting signal among datasets.

3.2.2 Phylogeny of Calliphorinae

Calliphorinae was consistently recovered as monophyletic and sister to Luciliinae. The former Melanomyinae were nested within Calliphorinae, supporting their synonymization (PP = 1.0; SH-aLRT = 99.9; UFB = 100.0). Within the clade, the Neotropical Toxotarsinae formed a basal lineage. The genus *Calliphora* was rendered paraphyletic in all analyses, with Australasian and Northern Hemisphere species forming two distinct clades separated by Melanomyinae + *Pericallimyia* Villeneuve. *Cynomya* clustered within *Calliphora*, particularly as sister to *Calliphora latifrons* Hough, reaffirming prior molecular findings (Stevens and Wall 2001; McDonagh and Stevens 2011). The monophyly of *Calliphora* was rejected by topology tests (AU, SH, KH) (**Parmar et al. 2025b:** Table S8).

3.2.3 Phylogeny of Luciliinae

Luciliinae was strongly supported as monophyletic and sister to Calliphorinae (PP = 1.0; SH-aLRT = 99.4; UFB = 100.0). However, the genus *Lucilia* was paraphyletic due to the nested placement of *Blepharicnema*, *Hemipyrellia*, and *Hypopygiopsis*. The monophyly of *Lucilia* was rejected by topology tests (AU, SH, KH) (**Parmar et al. 2025d, submitted:** Table S6).

Species-level relationships were well resolved. *Lucilia cuprina* and *L. sericata* formed a shallow clade with evidence of paraphyly. Additional clades included groups of *Lucilia eximia* Wiedemann, *Lucilia fayeae* Whitworth, *Lucilia retroversa* James and *Lucilia rica* Shannon, and Australasian-Palearctic clusters (e.g., *Lucilia bazini* Séguy, *Lucilia calviceps* Bezzi, *Lucilia hainanensis* Fan) (PP = 1.0; SH-aLRT = 76.2–98.1; UFB = 89–100.0). *Hemipyrellia* and *Hypopygiopsis* were consistently recovered within Luciliinae, with high sequence divergence between *Hypopygiopsis* clades. *Lucilia infernalis* Villeneuve was generally recovered as one of the two splits within the lineage as sister to other Luciliinae with strong nodal support across all analyses (PP = 1.0; SH-aLRT = 100.0; UFB = 100.0).

3.2.4 Geographic Patterns and Lineage Structure

Biogeographic structure was evident within Calliphoridae, especially in Calliphorinae and Luciliinae. Within Calliphorinae, the Neotropical representative from the former Toxotarsinae was consistently recovered as sister to all calliphorids. The largest genus, *Calliphora* was clearly separated into two clades: Northern Hemisphere, including *Cyanus*, *Cynomya* and *Cynomyiomima* and second clade consisting of the Australasian *Calliphora*, including species of the predominantly Palearctic, multilarviparous genera *Bellardia* Robineau-Desvoidy, *Onesia* Robineau-Desvoidy and *Polleniopsis* Townsend. Within Luciliinae, *Lucilia sericata* formed a globally distributed clade with minimal divergence, suggesting a recent expansion. *Lucilia cuprina* showed poor geographic resolution, with some Australian specimens clustering with *L. sericata*. *Lucilia porphyrina* Walker was paraphyletic with distinct Australasian and Southeast Asian clades. The Palaearctic *Lucilia caesar* Linnaeus and the Holarctic *Lucilia illustris* Meigen were clearly separated. *Hemipyrellia* and *Hypopygiopsis* exhibited clear Eastern Hemisphere and Australasian distributions, respectively.

3.3 Molecular Delimitation of Palaearctic and Afrotropical **Calliphora** *Robineau-Desvoidy and Descriptions of Two New Species from Ethiopia*

Our study identified and characterized two novel blowfly species from Ethiopia's Bale Mountains. *Calliphora teraramma* (**Parmar et al. 2025c**) is distinguished by its large body size (11–13 mm), broad frons, short antennae, and unique male genitalia, including elongated, fused cerci and a distinctive heavily sclerotized phallus with a broad ventral plate. Females of this species possess a remarkable feature—thick and long setae on the fifth tergite that project beyond the abdominal margin—unseen in related taxa (**Parmar et al. 2025c:** Fig. 1–3).

The second species, *Calliphora mesay* (**Parmar et al. 2025c**), closely resembles *C. vicina* but differs in key morphological traits such as dark parafacial and fronto-orbital plates, dark face and genal dilation, dark anterior thoracic spiracle, black basicosta, along with a broader male frons (**Parmar et al. 2025c:** Fig. 4–6). Both species were collected in high-altitude habitats, attracted to fish bait and human feces, suggesting similar ecological roles as decomposers and potential facultative parasites.

The two newly described species, *C. teraramma*, "ካሊፎራ ተራራማ" and *C. mesay*, "ካሊፎራ መሳይ" (**Parmar et al. 2025c**) are named using words derived from Amharic, a Semitic language widely spoken in Ethiopia, to honor the origin of these specimens and their first discovery in the region. The epithet *teraramma* combines “terara” (ተራራ), meaning mount or mountain, and “amma”, a feminine noun suffix or form of endearment, in reference to the Bale Mountains National Park, Sanetti Plateau, where the species was collected. This name pays tribute to the high-elevation mountainous distribution of this species.

The name *mesay* (መሳይ) translates to “look alike” or “similar” in Amharic, and reflects the species’ morphological resemblance to *Calliphora vicina*. The name was chosen to acknowledge this similarity while marking its distinctiveness as a newly discovered taxon. Both names are treated as nouns in apposition and reflect an effort to incorporate local language and cultural context in species nomenclature.

Barcode-based ML phylogenies and species delimitation for Palearctic and Afrotropical *Calliphora* and *Cynomya* supported most morphologically defined species. Notable exceptions included genetically distinct clades within *Calliphora loewi* Enderlein and *C. subalpina*, suggesting cryptic divergence or hybridization.

Newly described species, *C. teraramma* and *C. mesay*, formed distinct, well-supported lineages (> 80% SH-aLRT and UFB) (**Parmar et al. 2025c:** Fig. S1).

Molecular species delimitation analysis by ABGD and ASAP methods confirmed the taxonomic distinctness of *Calliphora teraramma*, showing a minimum 5.26% interspecific COI divergence from congeners. The maximum intraspecific distance was 1.52%, with a barcode gap of 3.74%–6.38%, supporting its status as a well-defined species under the DNA barcoding criterion (Hebert et al. 2003). In contrast, species delimitation analyses could not clearly resolve *C. mesay* as a distinct species, with minimal COI divergence between *C. mesay* and *C. vicina* (0.6–1.2%) (**Parmar et al. 2025c:** Fig. S2). However, whole mitogenome comparisons revealed 16 fixed nucleotide differences in COI and 98 across all mitochondrial protein-coding genes, supporting its status as a distinct species new to science.

3.4 Divergence Time Estimation and Historical Biogeography

Divergence time estimates from two independent MCMCtree runs were consistent, with overlapping posterior distributions (**Parmar et al. 2025d, submitted:** Fig. 3). The origin of Oestroidea was dated to ~37.4 Ma (95% CI: 36.6–38.4 Ma), and the core Calliphoridae began diverging around 23.0 Ma (95% CI: 21.1–25.2 Ma). Most calliphorid subfamilies diverged between ~20.8 Ma (95% CI: 18.48–23.24) and 15.9 Ma (95% CI: 14.63–17.27), the latter marking the origin of the Calliphorinae–Luciliinae sister lineage.

Within Luciliinae, genus-level diversification occurred between ~13.1 Ma and 5.6 Ma. In contrast, most within-species divergences were recent, generally postdating 4 Ma and concentrated in the Pliocene–Pleistocene. Shallow divergences (<0.2 Ma) were observed between *L. fayeae* and *L. rica*, *Hypopygiopsis infumata* Bigot and *H. violacea* Macquart, and *L. cuprina* and *L. sericata*. Most intraspecific splits occurred between 0.1–0.8 Ma, with deeper divergences in *Lucilia papuensis* Macquart and *L. porphyrina* (~4.6–5.6 Ma).

Geographic structuring was evident across lineages. Neotropical species (e.g., *Lucilia cluvia* Walker, *L. eximia*, *L. retroversa*) diversified during the late Miocene (5–9 Ma), while Palearctic and Nearctic species (*L. caesar*, *L. illustris*, *L. sericata*) showed more recent divergences (<2 Ma). The *L. cuprina–L. sericata* complex formed a shallow clade (~0.18–0.21 Ma), with the exception of a divergent Namibian *L. cuprina*

lineage (~1.87 Ma). Several Eastern Hemisphere tropical taxa showed deep divergence. *Lucilia infernalis*, endemic to Africa, represented one of the two lineages produced by the initial split of Luciliinae (~13.09 Ma), while *Hypopygiopsis* species from Southeast Asia diverged between ~6–13 Ma.

3.5 Evolutionary Transitions in Larval Feeding Modes

Ancestral-state reconstruction favored the Equal Rates (ER) model (AIC = 173.71; AIC weight = 0.51) and the Symmetrical (SYM) model (AIC = 173.80; AIC weight = 0.49) as nearly equally well-supported explanations of larval feeding mode evolution, suggesting uniform transition probabilities across states and no strong directional trends. This implies that feeding modes evolved flexibly, with multiple direct transitions between saprophagy, facultative parasitism, and obligate parasitism. Contrary to stepwise evolutionary models (Blaimer et al. 2020), the results indicate that lineages could shift feeding strategies without requiring intermediate stages.

Reconstructions inferred that early blowflies (around the Oligocene–Miocene transition, ~23 Ma) were likely obligate or facultative parasites (mean probabilities: 0.34 and 0.23, respectively), while the ancestral state of Luciliinae leaned toward saprophagy or facultative parasitism (mean probabilities: 0.51 and 0.45, respectively) (**Parmar et al. 2025d, submitted:** Fig. 3–4). Within Luciliinae, obligate parasitism evolved independently at least twice—in *Lucilia bufonivora* and *Lucilia elongata* Shannon—around the Pliocene (~4.2 Ma), whereas *L. infernalis*, representing one of the early splits within Luciliinae species (~13.1 Ma), remained saprophagous. Notably, key myiasis-causing species (*L. caesar, L. cuprina, L. illustris, L. sericata*) evolved independently in distinct clades across diverse climatic regions.

DISCUSSION

4.1 Mitogenomic Stability and Evolution

4.1.1 Conserved Mitochondrial Architecture and Composition

The highly conserved gene order and structure across Calliphoridae—especially in Luciliinae and Calliphorinae—reflect strong evolutionary constraints on mitogenome organization. However, significant subfamily-level differences observed in base composition and strand asymmetry suggest lineage-specific mutational pressures, possibly linked to differences in metabolic activity, ecological niches, or replication mechanisms (Lu et al. 2023; Kapoor et al. 2024; **Parmar et al. 2025b, Parmar et al. 2025d, submitted**).

These compositional biases had minimal impact on tree topology, reaffirming the reliability of mitogenomic datasets for phylogenetic reconstruction despite variation in AT/GC content.

4.1.2 Codon Usage: The Interplay Between Mutation and Selection

Codon usage patterns mirrored the AT-rich background of insect mitogenomes but also suggested selective optimization (Yan, Xu, et al. 2021; Lu et al. 2023; Kapoor et al. 2024). Codon–anticodon compatibility and the overrepresentation of codons for frequently used tRNAs indicate a balance between neutral mutational pressure and translational efficiency. This dual influence underscores the adaptive potential of mitochondrial genomes, even within a highly conserved framework (summarized in **Parmar et al. 2025b**).

4.1.3 Evolutionary Rates Reflect Ecological Strategies

Lineage-specific differences in substitution rates, particularly the elevated Ka and ω values in Ameniinae and Rhinophorinae, may reflect relaxed constraints or adaptive shifts, especially in lineages with parasitic or specialized ecologies (Lawrie et al. 2013). In contrast, Luciliinae and Calliphorinae appear to experience strong purifying selection, possibly due to broader ecological tolerances or more stable host interactions (Yan, Xu, et al. 2021; Shang et al. 2022).

The gene-level rate heterogeneity, especially in ATP8 and ND genes (ND2, ND5 and ND6), provides insights into regions of the mitogenome under relaxed

functional constraints or experiencing rapid evolution (Oliveira et al. 2008), which may be important for ecological adaptation.

4.1.4 Rearrangement Events are Rare and Lineage-Specific

The rarity and lineage-restriction of major gene rearrangements suggest that such events are independent and non-synapomorphic in Calliphoridae. The rrnL rearrangement in *C. varifrons* represents the first such report in the family and highlights the role of local repeats in mediating structural instability (**Parmar et al. 2025b**). Though structurally striking, these rearrangements do not appear to reflect shared evolutionary history and thus must be interpreted cautiously in phylogenetics.

The detection of divergent and duplicated/triplicated tRNAs—often within single species/populations—suggests a largely neutral, stochastic origin. Their sporadic distribution and lack of correlation with compositional or selective forces argue against functional or adaptive interpretations in most cases. Nonetheless, the potential for these duplicated tRNAs to affect translational dynamics or reflect subtle metabolic adaptations remains an area for further study.

4.2 Robust Phylogenetic Inference Across Calliphoridae

4.2.1 Phylogenetic Robustness and Data Treatments

Partitioned mitogenomic datasets recovered consistent topologies across ML and BI methods. Calliphoridae and its subfamilies were mostly monophyletic, confirming previous phylogenomic studies (Kutty et al. 2010; Nelson et al. 2012; Yan, Pape, et al. 2021; Shang et al. 2022; Cerretti et al. 2024). The placement of *Eurychaeta palpalis* (Ameniinae, former Helicoboscinae) remains unresolved and may reflect compositional bias, incomplete lineage sorting, or long-branch attraction (Degnan and Rosenberg 2009; Nelson et al. 2012; Sonet et al. 2012; Williams et al. 2016). RY coding and third codon position exclusion had little impact on overall topology but reduced nodal support, reinforcing the robustness of using full codon partitions for blowfly phylogenetics.

4.2.2 Evolutionary Insights within Calliphorinae and Luciliinae

The paraphyly of *Calliphora* supports previous findings and underscores the need for taxonomic revision. Our results suggest *Cynomya* should be synonymized with *Calliphora*, corroborated by molecular data, larval morphology, and wing morphometrics (Stevens and Wall 2001; Szpila 2009; Szpila et al. 2014; Szpila et al. 2019). However, adult morphological distinctions and low nodal support in some datasets warrant further integrative taxonomic work, incorporating detailed comparative morphology across life stages, nuclear genomic data, and expanded taxon sampling (especially for problematic genera such as *Cyanus* and *Cynomyiomima*).

The nested placement of former Melanomyinae within Calliphorinae reflects their shared evolutionary history, now supported by multiple datasets (morphology, UCEs, transcriptomics) (Rognes K. 1991; Buenaventura et al. 2021; Yan, Pape, et al. 2021). The basal position of Toxotarsinae may suggest a Southern Hemisphere origin for Calliphorinae.

The consistent recovery of *Lucilia* as paraphyletic, and the strong support for *Blepharicnema*, *Hemipyrellia*, and *Hypopygiopsis* within the clade, highlight ongoing instability at the genus level. Topology testing strongly rejected *Lucilia* monophyly. These results align with earlier molecular studies (Park et al. 2009; DeBry et al. 2010; McDonagh and Stevens 2011; Williams et al. 2016; Cardoso et al. 2025) and suggest that convergent morphologies or retained ancestral traits obscure true evolutionary relationships, complicating genus-level taxonomy in Luciliinae.

4.2.3 Biogeographic Patterns and Cryptic Diversity

Luciliinae and *Calliphora* lineages exhibit a clear geographic structure, reflecting historical dispersal, isolation, and possibly adaptation to different ecological niches (summarized in **Parmar et al. 2025b, Parmar et al. 2025d, submitted**). The shallow divergence of globally distributed *L. sericata* and the unresolved structure in *L. cuprina* suggest recent range expansion and possible hybridization (Stevens et al. 2002; Tourle et al. 2009; McDonagh and Stevens 2011; Kapoor et al. 2025). In contrast, deeper splits in *L. porphyrina* and *Hypopygiopsis* indicate older divergence events across Southeast Asia and Australasia. The placement of *Hemipyrellia* within *Lucilia* supports prior suggestions of synonymy and challenges its current generic status (Wells et al. 2007;

Park et al. 2009; McDonagh and Stevens 2011; Williams et al. 2016). The strong separation of *L. caesar* and *L. illustris* validates their status as distinct species, even though they are often sympatric across the Palaearctic and difficult to distinguish with mtDNA in some regions (DeBry et al. 2013). Likewise, the well-supported Western Hemisphere clade, including *L. coeruleiviridis* Macquart, *L. eximia*, *L. rica* and others, aligns with previous observations of regional endemism in the Americas (Wallman et al. 2005). The distinct placement of *L. infernalis* as one of the two basal lineages in our phylogeny, together with its Afrotropical endemism, is consistent with Africa having been part of the ancestral area of Luciliinae, with subsequent diversification of its sister lineage, comprising all other Luciliinae, across other geographic regions.

New barcoding and phylogenetic evidence also revealed cryptic species within *Calliphora*, including divergent clades in *C. loewi* and *C. subalpina*. This reinforces the value of integrating molecular data in taxonomic assessments, particularly in underexplored regions like the Afrotropics (**Parmar et al. 2025c**).

4.2.4 Implications and Future Directions

This study provides one of the most comprehensive mitogenome-based phylogenetic frameworks for Calliphoridae to date. Despite robust subfamily-level resolution, genus-level paraphyly and unstable placements of key taxa (e.g., *Cynomyiomima*, *E. palpalis*) highlight the limitations of mitochondrial data alone. Future work incorporating nuclear phylogenomics, site-heterogeneous models, and expanded taxon sampling will be essential for resolving these complex relationships and guiding taxonomic revision in this ecologically, medically and forensically important group.

4.3 Taxonomic and Evolutionary Implications of New **Calliphora** *Robineau-Desvoidy Species in the Afrotropics*

The description of *Calliphora teraramma* and *Calliphora mesay* addresses gaps in Afrotropical blowfly taxonomy while highlighting challenges in species delimitation (**Parmar et al. 2025c**). *Calliphora teraramma* represents a morphologically and genetically distinct lineage, with its unique genital architecture suggesting adaptive evolution in isolation. In contrast, *Calliphora mesay* exemplifies cryptic speciation, where negligible COI distances—common in recently diverged taxa (Wells et al. 2007; Whitworth et al. 2007; Sonet et al. 2012; Williams et al. 2016; Kapoor et al. 2023;

Johnston et al. 2025)—contrast with diagnostic morphological and mitogenomic differences. This discrepancy reinforces the necessity of integrative taxonomy, particularly in blowflies, where convergent morphology and rapid radiations complicate classification. The male genital traits of *C. teraramma* aligns it with *Calliphora genarum* Zetterstedt and *Calliphora grunini* Schumann & Ozerov, yet its derived features (e.g., upraised epiphallus) imply functional specialization, possibly linked to mating or oviposition strategy. Meanwhile, the close affinity of *C. mesay* with *C. vicina* mirrors global patterns of localized speciation in widespread taxa, potentially driven by Ethiopia's topographic complexity and climate fluctuations associated with Miocene–Pleistocene uplift events (Lyra et al. 2023). These findings underscore the Afrotropics as underexplored biodiversity hotspot and we advocate for expanded sampling across this region to resolve biogeographic and phylogenetic uncertainties in Calliphoridae.

4.4 Miocene Origins and Pleistocene Radiations: Geoclimatic Drivers of Luciliinae Evolution

Our divergence time estimates align with previous studies (e.g., Wiegmann et al. 2011; Junqueira et al. 2016), supporting a late Eocene origin of Oestroidea and an early Miocene diversification of the core Calliphoridae (**Parmar et al. 2025d, submitted**). This early radiation likely coincided with global environmental changes, including increased aridification, the expansion of grasslands, and the diversification of large herbivorous mammals, particularly even-toed ungulates (Stebbins 1981; Bredenkamp et al. 2002). These factors likely boosted carrion availability, facilitating ecological expansion and diversification in blowflies (Junqueira et al. 2016). Geophysical changes such as the closure of seaways between Africa and Eurasia, and the persistence of the Bering land bridge, further enabled intercontinental faunal exchange and may have shaped early biogeographic patterns (Zachos et al. 2001).

Compared to earlier studies with limited Luciliinae sampling (e.g., Wallman et al. 2005; Wiegmann et al. 2011; Junqueira et al. 2016; Cerretti et al. 2017; Arias-Robledo et al. 2019), our expanded dataset provided a more detailed view of the subfamily's evolutionary history. Luciliinae likely originated during the Middle Miocene (~15–20 Ma), consistent with prior estimates (e.g., Wallman et al. 2005; Wiegmann et al. 2011; Junqueira et al. 2016; Cerretti et al. 2017; Arias-Robledo et al.

2019). This timeframe overlaps with the Middle Miocene Climatic Optimum (MMCO), a period of warming and biome restructuring that may have promoted diversification into parasitic and saprophagous niches. Geological events such as the uplift of major mountain ranges and the expansion of savannas likely contributed to early allopatric speciation.

Our results suggest Africa having been part of the early range of Luciliinae, with *Lucilia infernalis* representing one of the two the contemporaneous lineages (~13.1 Ma), followed by dispersal to Asia and the Western Hemisphere. Most deep divergences within the subfamily (~6–13 Ma) occurred in Eastern Hemisphere lineages, while Western Hemisphere radiations appeared more recently, following Miocene and Pliocene environmental transitions.

Lineage divergences corresponding to the currently recognized genera were clustered in the Miocene, whereas most intraspecific divergence took place during the Pliocene and Pleistocene, likely driven by glacial cycles, habitat fragmentation, and climatic fluctuations. Recent divergences in species complexes such as *L. cuprina–L. sericata* point to shallow splits (<0.2 Ma), likely influenced by human activity, synanthropy, and possible ongoing gene flow or hybridization (Erzinçlioğlu 1989; Stevens and Wall 1997; Wallman et al. 2005; Stevens et al. 2006; Kapoor et al. 2025). In such cases, species boundaries may be blurred at the molecular level, as recent divergence and genetic exchange can obscure phylogenetic signal and lead to incomplete lineage sorting. This highlights the importance of integrative species delimitation approaches that combine molecular, morphological, ecological, and behavioral data to accurately distinguish closely related, morphologically similar, and medically relevant taxa.

Deeper splits observed in the Namibian *L. cuprina* lineage (~1.87 Ma) and between *L. papuensis* and *L. porphyrina* may reflect incomplete lineage sorting, introgression, or overlooked cryptic diversity. Neotropical taxa, including *L. cluvia*, *L. eximia* and *L. retroversa*, diverged during the late Miocene (5–9 Ma), coinciding with major geological events such as the Andean uplift and Panama Isthmus closure (~3 Ma), suggesting a role for elevational and dispersal barriers in speciation (Bermingham and Martin 1998).

In contrast, Palearctic and Nearctic species (*L. caesar*, *L. illustris*, *L. sericata*) diverged more recently (<2 Ma), likely in response to Pleistocene glaciations, which

drove repeated cycles of expansion and contraction, isolation in glacial refugia, and subsequent divergence between Eurasia and North America (Markgraf et al. 1995).

Collectively, these findings underscore the influence of Miocene–Pleistocene geoclimatic events in shaping Luciliinae diversity, supporting a model of region-specific radiations driven by ecological opportunity, environmental change, and vicariant processes.

4.5 Ancestral State Reconstruction revealed multiple independent origins and homogeneous transitions of larval feeding strategies in Luciliinae

Our findings challenge the traditional view of a linear progression from saprophagy to obligate parasitism via facultative stages (Zumpt 1965; Erzinçlioğlu 1989; Stevens and Wall 1997). Instead, larval feeding strategies in Luciliinae and the broader Calliphoridae evolved through multiple independent transitions, with no strict directional constraint (**Parmar et al. 2025d, submitted**).

The early Miocene saw the emergence of diverse feeding strategies, coinciding with expanded ecological niches following the proliferation of gastropods, arthropods, and mammals (Solórzano Kraemer et al. 2015; Höltke et al. 2016; Grossmann et al. 2023). Several parasitic lineages in other calliphorid subfamilies (e.g., Ameniinae, Bengaliinae, Rhiniinae) likely originated in response to host availability during this period.

Within Luciliinae, obligate parasitism evolved in specific lineages (e.g., *L. bufonivora*), while most tropical taxa retained saprophagy, suggesting that parasitism emerged in response to ecological pressures—such as host abundance or carrion competition—rather than geography alone (Strömberg 2011; Steinthorsdottir et al. 2021). These transitions often coincided with major climatic shifts—such as the Middle Miocene Climatic Optimum and Pliocene cooling—which likely shaped niche availability. Our results also reinforce previous suggestions that parasitic traits are phylogenetically clustered, not geographically structured, and that parasitism in Luciliinae evolved multiple times independently.

In summary, larval feeding strategies in Calliphoridae, particularly Luciliinae, reflect a dynamic and reversible evolutionary history, driven by ecological opportunity and lineage-specific responses to changing environments. Broader taxonomic and

genomic sampling will be essential to fully resolve the ancestral states and evolutionary drivers of parasitism in blowflies.

CONCLUSION

This thesis presents the most comprehensive mitogenomic and phylogenetic investigation of Calliphoridae to date, with an emphasis on Luciliinae and Calliphorinae, two of the most medically, forensically, veterinary and ecologically significant subfamilies. By assembling and analyzing the largest mitogenomic dataset ever compiled for blowflies—encompassing 107 newly sequenced species across all major subfamilies from diverse geography—this work has resolved long-standing taxonomic uncertainties, revealed hidden diversity, and offered foundational insights into mitochondrial genome evolution in blowflies (**Parmar et al. 2025b, 2025c and Parmar et al. 2025d, submitted**).

Comparative mitogenomic analyses, particularly within Calliphorinae offer new insights into mitochondrial genome architecture, revealing diverse structural and compositional mitochondrial features across subfamilies (**Parmar et al. 2025b**). While rearrangements were rare and mostly confined to Calliphorinae, notable exceptions such as those in *C. varifrons* may reflect lineage-specific dynamics and speciation-related events within the subfamily. Codon usage bias, tRNA duplications, and strand asymmetry patterns provide additional evidence of selective and mutational forces shaping mitochondrial evolution. Importantly, the relatively slow evolutionary rate of Calliphorinae mitogenomes suggests strong purifying selection, likely linked to the ecological specialization within this group.

Our comprehensive phylogenetic framework supports monophyletic Calliphoridae and validates Rhiniinae and Rhinophorinae as calliphorid subfamilies (**Parmar et al. 2025b, Parmar et al. 2025d, submitted**). We also provide strong support for including the former Melanomyinae and Toxotarsinae within Calliphorinae. Notably, *Pericallimyia* is recovered within Calliphorinae, as sister to Melanomyinae, supporting recent synonymization of Melanomyinae with Calliphorinae. The basal position of Toxotarsinae hints at a Southern Hemisphere origin of Calliphorinae. The largest genus, *Calliphora* is confirmed as paraphyletic, with implications for potential synonymization with *Cyanus*, *Cynomya*, and *Cynomyiomima*. Similarly, in Luciliinae, the paraphyly of *Lucilia*—with *Blepharicnema*, *Hemipyrellia*, and *Hypopygiopsis* nested within—demands future taxonomic revision.

Divergence dating and ancestral state reconstructions place the origin of Luciliinae in during the mid-Miocene, with tropical Africa as probable ancestral area, and suggest that subsequent diversification was shaped by climatic shifts and biogeographic dispersal (**Parmar et al. 2025d, submitted**). Contrary to a stepwise

model of myiasis evolution, this study reveals flexible, reversible transitions in larval feeding strategies, with facultative parasitism acting as a key adaptive state in response to ecological opportunity. Independent origins of obligate parasitism in *Lucilia bufonivora* and *L. elongata* highlight the evolutionary plasticity of trophic strategies in blowflies, which has direct implications for understanding the emergence and spread of myiasis-causing species.

The discovery and formal description of two novel *Calliphora* species from Ethiopia i.e., *C. teraramma* and *C. mesay* expands our understanding of Afrotropical diversity and demonstrates the utility of integrating mitochondrial genomics with detailed morphological diagnosis for species delimitation in morphologically similar taxa (**Parmar et al. 2025c**). The updated DNA barcoded library for Afrotropical and Palaearctic *Calliphora* enhance regional biodiversity knowledge and are particularly relevant to forensic entomology, where accurate species identification is critical for estimating postmortem intervals, and to medical and veterinary entomology, where species-specific parasitism plays a role in wound myiasis and disease transmission.

In summary, this thesis underscores the power of mitogenomic data for elucidating evolutionary histories in dipteran lineages and supporting practical applications in forensic, medical, and veterinary contexts. While mitogenomes offer a powerful toolset, our findings also underscore the importance of integrative approaches—combining mitochondrial and nuclear markers with morphological and ecological data, especially for resolving higher-level relationships and recent divergences. Nevertheless, by bridging taxonomy, systematics, and evolutionary biology, this work provides a robust framework for future investigations into the diversification, adaptation, and ecological roles of blowflies, particularly in the face of shifting climates and emerging parasitic threats.

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TABLE T1

Table T1: Summary of the specimens used for mitochondrial genome assembly in the present study. Specimen IDs beginning with 'KEIB_DIP' represent those sequenced as part of this study. Species labelled with '*' are represented by incomplete mitochondrial genomes. Species names in bold denote newly described taxa, published as part of this thesis (Parmar et al. 2025c). Detailed locations of specimen collection are provided in Table S1 (Parmar et al. 2025b, 2025c and 2025d, submitted).

ID	Family	Subfamily	Genera	Species	Country	Lat.	Long.	GenBank Accession Numbers	BOLD Accession Numbers
KEIB_DIP_02009	Calliphoridae	Ameniinae	*Amenia*	*A. imperialis*	Australia	-35.205	150.539	PQ665281	KEIB160-24
KEIB_DIP_02012	Calliphoridae	Ameniinae	*Paramenia*	*P. semiauriceps*	Australia	-34.634	150.727	PQ665282	KEIB161-24
KEIB_DIP_02017	Calliphoridae	Ameniinae	*Eurychaeta*	*E. palpalis*	Poland	49.222	22.55	PQ665283	KEIB162-24
KEIB_DIP_01983	Calliphoridae	Bengaliinae	*Tricyclea*	*Tricyclea* sp. 2	Ethiopia	6.011	37.559	PQ654328	KEIB163-24
KEIB_DIP_01980	Calliphoridae	Bengaliinae	*Cordylobia*	*C. anthropophaga*	Ethiopia	6.033	37.559	PQ654329	KEIB164-24
KEIB_DIP_01981	Calliphoridae	Bengaliinae	*Bengalia*	*B. seniorwhitei*	Ethiopia	6.011	37.559	PQ654330	KEIB165-24
KEIB_DIP_02070	Calliphoridae	Calliphorinae	*Calliphora*	*Calliphora* sp. n. 3	Portugal	32.802	-17.166	PQ593971	KEIB001-24
KEIB_DIP_02099	Calliphoridae	Calliphorinae	*Calliphora*	*C. alaskensis*	Canada	49.374	-123.243	PQ593972	KEIB002-24
KEIB_DIP_02101	Calliphoridae	Calliphorinae	*Calliphora*	*C. augur**	Australia	-35.275	149.097	Appendix 1	KEIB003-24
KEIB_DIP_02102	Calliphoridae	Calliphorinae	*Calliphora*	*C. calliphoroides*	Russia	51.38	105.17	PQ593973	KEIB004-24
KEIB_DIP_02104	Calliphoridae	Calliphorinae	*Calliphora*	*C. coloradensis*	U.S.A.	42.804	-118.866	PQ593974	KEIB005-24
KEIB_DIP_02109	Calliphoridae	Calliphorinae	*Calliphora*	*C. genarum*	Russia	51.403	105.08	PQ593975	KEIB006-24
KEIB_DIP_02110	Calliphoridae	Calliphorinae	*Calliphora*	*C. grahami*	North Korea	38.689	128.18	PQ593976	KEIB007-24
KEIB_DIP_02112	Calliphoridae	Calliphorinae	*Calliphora*	*C. hilli*	Australia	-32.964	150.435	PQ593977	KEIB008-24
KEIB_DIP_02113	Calliphoridae	Calliphorinae	*Calliphora*	*C. himalayana*	India	34.08	74.349	PQ593978	KEIB009-24
KEIB_DIP_02115	Calliphoridae	Calliphorinae	*Calliphora*	*C. livida**	U.S.A.	43.03	-87.88	Appendix 1	KEIB010-24
KEIB_DIP_02116	Calliphoridae	Calliphorinae	*Calliphora*	*C. livida**	U.S.A.	44.819	-117.892	Appendix 1	KEIB011-24
KEIB_DIP_02121	Calliphoridae	Calliphorinae	*Calliphora*	*C. montana**	Russia	63.8	137.883	Appendix 1	KEIB013-24
KEIB_DIP_02122	Calliphoridae	Calliphorinae	*Calliphora*	*C. calliphoroides*	Japan	35.866	139.623	PQ594007	KEIB014-24
KEIB_DIP_02123	Calliphoridae	Calliphorinae	*Calliphora*	*C. nigribarbis**	Japan	35.619	139.685	Appendix 1	KEIB015-24

KEIB_DIP_02124	Calliphoridae	Calliphorinae	*Calliphora*	*C. nigrithorax*	Australia	-34.79	150.776	PQ593979	KEIB016-24
KEIB_DIP_02130	Calliphoridae	Calliphorinae	*Calliphora*	*C. rohdendorfi*	Russia	44.534	38.568	PQ593980	KEIB017-24
KEIB_DIP_02131	Calliphoridae	Calliphorinae	*Calliphora*	*C. splendens* *	Spain	28.264	-16.74	Appendix 1	KEIB018-24
KEIB_DIP_02132	Calliphoridae	Calliphorinae	*Calliphora*	*C. stelviana* *	Sweden	63.317	12.102	Appendix 1	KEIB019-24
KEIB_DIP_02135	Calliphoridae	Calliphorinae	*Calliphora*	*C. subalpina*	Poland	49.394	20.395	PQ593981	KEIB020-24
KEIB_DIP_02136	Calliphoridae	Calliphorinae	*Calliphora*	*C. subalpina*	Russia	51.403	105.08	PQ593982	KEIB021-24
KEIB_DIP_02137	Calliphoridae	Calliphorinae	*Calliphora*	*C. subalpina* *	Russia	51.336	104.681	Appendix 1	KEIB022-24
KEIB_DIP_02138	Calliphoridae	Calliphorinae	*Calliphora*	*C. terraenovae*	U.S.A.	46.972	-118.615	PQ594006	KEIB023-24
KEIB_DIP_02140	Calliphoridae	Calliphorinae	*Calliphora*	*C. uralensis* *	Poland	54.222	22.819	PQ594004	KEIB024-24
KEIB_DIP_02142	Calliphoridae	Calliphorinae	*Calliphora*	*C. vicina*	Iran	30.196	57.436	PQ593983	KEIB025-24
KEIB_DIP_02143	Calliphoridae	Calliphorinae	*Calliphora*	*C. varifrons*	Australia	-34.414	117.957	PQ594010	KEIB026-24
KEIB_DIP_02145	Calliphoridae	Calliphorinae	*Calliphora*	*C. zaidamensis**	Russia	51.787	87.253	PQ594005	KEIB027-24
KEIB_DIP_02146	Calliphoridae	Calliphorinae	*Calliphora*	***C. teraramma***	Ethiopia	6.849	39.892	PQ593984	KEIB028-24
KEIB_DIP_02147	Calliphoridae	Calliphorinae	*Calliphora*	***C. mesay***	Ethiopia	6.909	39.917	PQ593985	KEIB029-24
KEIB_DIP_02148	Calliphoridae	Calliphorinae	*Calliphora*	***C. mesay***	Ethiopia	6.909	39.917	PQ593986	KEIB030-24
KEIB_DIP_02150	Calliphoridae	Calliphorinae	*Calliphora*	***C. teraramma***	Ethiopia	6.849	39.892	PQ593987	KEIB031-24
KEIB_DIP_02151	Calliphoridae	Calliphorinae	*Calliphora*	***C. teraramma***	Ethiopia	7.11	39.764	PQ593988	KEIB032-24
KEIB_DIP_01527	Calliphoridae	Calliphorinae	*Calliphora*	*C. vicina*	Poland	50.901	20.88	PQ593989	KEIB033-24
KEIB_DIP_01540	Calliphoridae	Calliphorinae	*Calliphora*	*C. vomitoria*	Poland	50.836	21.109	PQ593990	KEIB034-24
KEIB_DIP_01711	Calliphoridae	Calliphorinae	*Calliphora*	*C. latifrons*	U.S.A.	35.708	-117.884	PQ593991	KEIB035-24
KEIB_DIP_01714	Calliphoridae	Calliphorinae	*Calliphora*	*C. triseta**	Costa Rica	9.56	-83.69	Appendix 1	KEIB036-24
KEIB_DIP_01712	Calliphoridae	Calliphorinae	*Calliphora*	*C. irazuana*	Costa Rica	9.56	-83.69	PQ593992	KEIB037-24
KEIB_DIP_01672	Calliphoridae	Calliphorinae	*Calliphora*	*C. uralensis*	Russia	66.8	65.8	PQ593993	KEIB038-24
KEIB_DIP_01571	Calliphoridae	Calliphorinae	*Calliphora*	*C. loewi*	Poland	50.692	19.414	PQ594008	KEIB039-24
KEIB_DIP_02015	Calliphoridae	Calliphorinae	*Calliphora*	*C. albifrontalis*	Australia	-34.414	117.957	PQ593994	KEIB040-24
KEIB_DIP_02018	Calliphoridae	Calliphorinae	*Calliphora*	*C. rohdendorfi*	Poland	50.476	16.734	PQ593995	KEIB041-24
KEIB_DIP_02026	Calliphoridae	Calliphorinae	*Calliphora*	*C. ochracea*	Australia	-34.79	150.776	PQ593996	KEIB042-24
KEIB_DIP_02027	Calliphoridae	Calliphorinae	*Calliphora*	*C. dubia*	Australia	-34.414	117.957	PQ593997	KEIB043-24

KEIB_DIP_02029	Calliphoridae	Calliphorinae	*Calliphora*	*C. quadrimaculata*	New Zealand	-45.73	167.36	PQ593998	KEIB044-24
KEIB_DIP_02030	Calliphoridae	Calliphorinae	*Xenocalliphora*	*X. neozelandica*	New Zealand	-45.73	167.36	PQ593999	KEIB045-24
KEIB_DIP_02032	Calliphoridae	Calliphorinae	*Calliphora*	*C. hilli*	Australia	-34.403	150.857	PQ594000	KEIB046-24
KEIB_DIP_02033	Calliphoridae	Calliphorinae	*Calliphora*	*C. sternalis*	Australia	-34.149	151.03	PQ594001	KEIB047-24
KEIB_DIP_01463	Calliphoridae	Calliphorinae	*Calliphora*	*C. subalpina*	Germany	48.437	9.724	PQ594009	KEIB048-24
KEIB_DIP_02043	Calliphoridae	Calliphorinae	*Calliphora*	*C. croceipalpis*	Ethiopia	9.086	38.733	PQ594002	KEIB049-24
KEIB_DIP_02085	Calliphoridae	Calliphorinae	*Onesia*	*O. zumpti*	Germany	48.906	9.068	PQ594003	KEIB050-24
KEIB_DIP_01557	Calliphoridae	Calliphorinae	*Onesia*	*O. floralis*	Poland	53.303	18.373	PQ635404	KEIB051-24
KEIB_DIP_01619	Calliphoridae	Calliphorinae	*Onesia*	*O. accepta*	Australia	-35.254	149.106	PQ635405	KEIB052-24
KEIB_DIP_02016	Calliphoridae	Calliphorinae	*Melinda*	*M. viridicyanea*	Poland	49.222	22.55	PQ635406	KEIB053-24
KEIB_DIP_02036	Calliphoridae	Calliphorinae	*Onesia*	*O. tibialis*	Australia	-34.403	150.857	PQ635407	KEIB054-24
KEIB_DIP_02037	Calliphoridae	Calliphorinae	*Onesia*	*O. minor*	Australia	-35.394	149.009	PQ635408	KEIB055-24
KEIB_DIP_02038	Calliphoridae	Calliphorinae	*Onesia*	*O. austriaca*	Poland	50.685	21.795	PQ635409	KEIB056-24
KEIB_DIP_02039	Calliphoridae	Calliphorinae	*Onesia*	*O. floralis*	Poland	53.117	17.4	PQ635410	KEIB057-24
KEIB_DIP_02091	Calliphoridae	Calliphorinae	*Bellardia*	*B. bayeri*	Iran	35.737	51.273	PQ635411	KEIB058-24
KEIB_DIP_02093	Calliphoridae	Calliphorinae	*Bellardia*	*B. pubicornis*	Sweden	63.677	13.22	PQ635412	KEIB059-24
KEIB_DIP_02095	Calliphoridae	Calliphorinae	*Bellardia*	*B. siciliensis*	Tunisia	36.783	8.687	PQ635413	KEIB060-24
KEIB_DIP_02096	Calliphoridae	Calliphorinae	*Bellardia*	*B. vespillo**	Poland	53.303	18.373	Appendix 1	KEIB061-24
KEIB_DIP_01528	Calliphoridae	Calliphorinae	*Bellardia*	*B. vulgaris*	Poland	50.901	20.88	PQ664927	KEIB062-24
KEIB_DIP_02013	Calliphoridae	Calliphorinae	*Bellardia*	*B. viarum*	Poland	50.692	19.414	PQ664928	KEIB064-24
KEIB_DIP_02014	Calliphoridae	Calliphorinae	*Bellardia*	*B. pandia*	Poland	50.692	19.414	PQ664929	KEIB065-24
KEIB_DIP_02072	Calliphoridae	Calliphorinae	*Cyanus*	*C. elongata*	U.S.A.	43.278	-118.806	PQ664930	KEIB066-24
KEIB_DIP_02074	Calliphoridae	Calliphorinae	*Cynomya*	*C. cadaverina*	U.S.A.	47.657	-117.28	PQ664931	KEIB067-24
KEIB_DIP_02028	Calliphoridae	Calliphorinae	*Cynomya*	*C. mortuorum*	Poland	52.564	19.678	PQ664986	KEIB068-24
KEIB_DIP_01969	Calliphoridae	Calliphorinae	*Pericallimyia*	*Pericallimyia* sp.	Ethiopia	7.116	39.738	PQ665286	KEIB069-24
KEIB_DIP_02088	Calliphoridae	Calliphorinae	*Polleniopsis*	*P. mongolica**	Russia	56.037	37.235	Appendix 1	KEIB070-24
KEIB_DIP_02089	Calliphoridae	Calliphorinae	*Polleniopsis*	*P. zaitzevi**	Sri Lanka	6.95	80.79	Appendix 1	KEIB071-24
KEIB_DIP_02076	Calliphoridae	Calliphorinae	*Cynomyiomima*	*C. stackelbergi**	Mongolia	46.355	97.359	Appendix 1	KEIB072-24

KEIB_DIP_02020	Calliphoridae	Calliphorinae	*Melinda*	*M. viridicyanea*	Poland	50.892	16.694	PQ664953	KEIB073-24
KEIB_DIP_02090	Calliphoridae	Calliphorinae	*Tricycleopsis*	*T. paradoxa**	Japan	32.756	129.873	Appendix 1	KEIB074-24
KEIB_DIP_02023	Calliphoridae	Calliphorinae	*Paradichosia*	*P. okazaki*	Russia	42.908	132.726	PQ664954	KEIB075-24
KEIB_DIP_01822	Calliphoridae	Calliphorinae	*Eggisops*	*E. pecchiolii*	Germany	48.437	9.724	PQ664955	KEIB076-24
KEIB_DIP_02041	Calliphoridae	Calliphorinae	*Sarconesia*	*S. magellanica*	Argentina	-26.07	-65.89	PQ649436	KEIB077-24
KEIB_DIP_02118	Calliphoridae	Calliphorinae	*Calliphora*	*C. loewi*	Russia	51.38	105.17	PQ654167	KEIB012-24
KEIB_DIP_02128	Calliphoridae	Calliphorinae	*Calliphora*	*C. peruviana**	Colombia	7.287	-76.098	Appendix 1	NA
KEIB_DIP_01594	Calliphoridae	Calliphorinae	*Bellardia*	*B. stricta*	Poland	52.977	18.581	PQ654168	KEIB063-24
KEIB_DIP_02268	Calliphoridae	Chrysomyinae	*Cochliomyia*	*C. minima*	Puerto Rico	18.405	-66.052	PQ649440	KEIB129-24
KEIB_DIP_02272	Calliphoridae	Chrysomyinae	*Compsomyiops*	*C. fulvicrura*	Argentina	-51.622	-69.22	PQ664987	KEIB131-24
KEIB_DIP_01559	Calliphoridae	Chrysomyinae	*Chrysomya*	*C. albiceps*	Poland	50.692	19.414	PQ665287	KEIB144-24
KEIB_DIP_01987	Calliphoridae	Luciliinae	*Hemipyrellia*	*H. fernandica*	Ethiopia	6.011	37.559	PQ649437	KEIB101-24
KEIB_DIP_01636	Calliphoridae	Luciliinae	*Lucilia*	*L. porphyrina*	Australia	-34.567	150.673	PQ649438	KEIB114-24
KEIB_DIP_02007	Calliphoridae	Luciliinae	*Lucilia*	*L. eximia*	French Guiana	4.628	-52.307	PQ649439	KEIB120-24
KEIB_DIP_02153	Calliphoridae	Luciliinae	*Blepharicnema*	*B. splendens*	Venezuela	10.416	-67.616	PV604281	KEIB078-24
KEIB_DIP_02161	Calliphoridae	Luciliinae	*Hypopygiopsis*	*H. tumrasvini*	Thailand	18.818	98.892	PV604282	KEIB082-24
KEIB_DIP_02171	Calliphoridae	Luciliinae	*Lucilia*	*L. cluvia*	U.S.A.	27.243	-80.826	PV604283	KEIB085-24
KEIB_DIP_02176	Calliphoridae	Luciliinae	*Lucilia*	*L. elongata*	U.S.A.	42.224	-121.78	PV604284	KEIB088-24
KEIB_DIP_02178	Calliphoridae	Luciliinae	*Lucilia*	*L. fayeae*	Dominican Republic	15.589	-61.354	PV604285	KEIB089-24
KEIB_DIP_02180	Calliphoridae	Luciliinae	*Lucilia*	*L. infernalis*	Malawi	-13.384	34.005	PV604286	KEIB091-24
KEIB_DIP_02187	Calliphoridae	Luciliinae	*Lucilia*	*L. papuensis*	Australia	-19.092	146.27	PV604287	KEIB093-24
KEIB_DIP_02188	Calliphoridae	Luciliinae	*Lucilia*	*L. pilosiventris*	Poland	50.421	20.645	PV604288	KEIB094-24
KEIB_DIP_02195	Calliphoridae	Luciliinae	*Lucilia*	*L. rica*	Antigua	17.08	-61.799	PV604289	KEIB096-24
KEIB_DIP_02200	Calliphoridae	Luciliinae	*Lucilia*	*L. sinensis*	Thailand	7.742	98.781	PV604290	KEIB098-24
KEIB_DIP_02201	Calliphoridae	Luciliinae	*Lucilia*	*L. shenyangensis*	India	30.461	78.067	PV604291	KEIB099-24
KEIB_DIP_02202	Calliphoridae	Luciliinae	*Lucilia*	*L. thatuna*	U.S.A.	37.685	-106.359	PV604292	KEIB100-24
KEIB_DIP_02045	Calliphoridae	Luciliinae	*Hemipyrellia*	*H. ligurriens*	Malaysia	3.39	101.78	PV604293	KEIB102-24
KEIB_DIP_01811	Calliphoridae	Luciliinae	*Hypopygiopsis*	*H. violacea*	Malaysia	2.029	102.984	PV604294	KEIB103-24

KEIB_DIP_01685	Calliphoridae	Luciliinae	*Hypopygiopsis*	*H. fumipennis*	Malaysia	3.39	101.78	PV604295	KEIB104-24
KEIB_DIP_01676	Calliphoridae	Luciliinae	*Hypopygiopsis*	*H. infumata*	Malaysia	3.39	101.78	PV604296	KEIB105-24
KEIB_DIP_01533	Calliphoridae	Luciliinae	*Lucilia*	*L. sericata*	Poland	50.901	20.88	PV604297	KEIB106-24
KEIB_DIP_01534	Calliphoridae	Luciliinae	*Lucilia*	*L. richardsi*	Poland	50.901	20.88	PV604298	KEIB107-24
KEIB_DIP_01535	Calliphoridae	Luciliinae	*Lucilia*	*L. silvarum*	Poland	50.901	20.88	PV604299	KEIB108-24
KEIB_DIP_01537	Calliphoridae	Luciliinae	*Lucilia*	*L. bufonivora*	Poland	50.827	21.03	PV604300	KEIB109-24
KEIB_DIP_01538	Calliphoridae	Luciliinae	*Lucilia*	*L. ampullacea*	Poland	50.836	21.109	PV604301	KEIB110-24
KEIB_DIP_01567	Calliphoridae	Luciliinae	*Lucilia*	*L. illustris*	Poland	50.692	19.414	PV604302	KEIB112-24
KEIB_DIP_01621	Calliphoridae	Luciliinae	*Lucilia*	*L. cuprina*	Australia	-34.403	150.879	PV604303	KEIB113-24
KEIB_DIP_01687	Calliphoridae	Luciliinae	*Lucilia*	*L. porphyrina*	Malaysia	3.39	101.78	PV604304	KEIB115-24
KEIB_DIP_01829	Calliphoridae	Luciliinae	*Lucilia*	*L. pilosiventris*	Germany	51.308	10.096	PV604305	KEIB116-24
KEIB_DIP_01731	Calliphoridae	Luciliinae	*Lucilia*	*L. pulverulenta*	Costa Rica	8.93	-82.78	PV604306	KEIB117-24
KEIB_DIP_01828	Calliphoridae	Luciliinae	*Lucilia*	*L. regalis*	Poland	53.303	18.373	PV604307	KEIB118-24
KEIB_DIP_02006	Calliphoridae	Luciliinae	*Lucilia*	*L. albofusca*	French Guina	4.559	-52.207	PV604308	KEIB119-24
KEIB_DIP_02031	Calliphoridae	Luciliinae	*Lucilia*	*L. sinensis*	Vietnam	20.351	105.593	PV604309	KEIB121-24
KEIB_DIP_02165	Calliphoridae	Luciliinae	*Lucilia*	*L. bazini**	Japan	26.231	127.759	PV604334	KEIB083-24
KEIB_DIP_02170	Calliphoridae	Luciliinae	*Lucilia*	*L. calviceps**	Papua New Guinea	-3.317	151.999	PV604335	KEIB084-24
KEIB_DIP_02175	Calliphoridae	Luciliinae	*Lucilia*	*L. cuprina**	Namibia	-23.541	15.296	PV604336	KEIB087-24
KEIB_DIP_01542	Calliphoridae	Luciliinae	*Lucilia*	*L. caesar*	Poland	50.836	21.109	PV604337	KEIB111-24
KEIB_DIP_02174	Calliphoridae	Luciliinae	*Lucilia*	*L. coeruleiviridis**	U.S.A.	40.412	-92.77	PV604341	KEIB086-24
KEIB_DIP_02157	Calliphoridae	Luciliinae	*Hemipyrellia*	*H. ligurriens**	Australia	-12.397	130.935	Appendix 1	KEIB080-24
KEIB_DIP_02194	Calliphoridae	Luciliinae	*Lucilia*	*L. retroversa**	Dominican Republic	18.802	-69.314	Appendix 1	KEIB095-24
KEIB_DIP_02000	Mesembrinellidae	Mesembrinellinae	*Mesembrinella*	*M. benoisti*	French Guiana	4.559	-52.207	PQ654166	NA
KEIB_DIP_01707	Calliphoridae	Phumosinae	*Phumosia*	*P. promittens*	Malaysia	3.39	101.78	PQ654331	KEIB166-24
KEIB_DIP_01692	Calliphoridae	Rhiniinae	*Rhyncomya*	*R. zumpti*	Namibia	-21.903	16.381	PQ654332	KEIB168-24
KEIB_DIP_01541	Calliphoridae	Rhinophorinae	*Rhinomorinia*	*R. sarcophagina*	Poland	50.836	21.109	PQ654169	KEIB167-24
KEIB_DIP_02069	Sarcophagidae	…	*Nephochaetopteryx*	*N. orbitalis*	French Guiana	4.559	-52.207	PQ654170	NA
KEIB_DIP_01708	Ulurumyiidae	…	*Ulurumyia*	*U. macalpinei*	Australia	-33.657	150.273	PQ654165	NA

MK591038	Calliphoridae	Bengaliinae	*Bengalia*	*Bengalia* sp.	China	18.706	108.928	MK591038	NA
KP872701	Calliphoridae	Calliphorinae	*Calliphora*	*C. grahami*	China	28.26	112.98	KP872701	NA
OU696535	Calliphoridae	Calliphorinae	*Bellardia*	*B. pandia*	U.K.	51.769	-1.339	OU696535	NA
OX493271	Calliphoridae	Calliphorinae	*Bellardia*	*B. bayeri*	U.K.	51.769	-1.328	OX493271	NA
OY288238	Calliphoridae	Calliphorinae	*Calliphora*	*C. vicina*	U.K.	51.884	-0.369	OY288238	NA
MT017724	Calliphoridae	Calliphorinae	*Calliphora*	*C. nigribarbis*	China	35.408	116.575	MT017724	NA
MT017707	Calliphoridae	Calliphorinae	*Calliphora*	*C. sinensis*	China	35.408	116.575	MT017707	NA
MT017722	Calliphoridae	Calliphorinae	*Calliphora*	*C. uralensis*	China	35.408	116.575	MT017722	NA
MT628574	Calliphoridae	Calliphorinae	*Cynomya*	*C. mortuorum*	Denmark	55.674	12.566	MT628574	NA
MN131052	Calliphoridae	Calliphorinae	*Polleniopsis*	*P. mongolica*	China	28.251	112.958	MN131052	NA
MK893471	Calliphoridae	Calliphorinae	*Calliphora*	*C. calliphoroides*	South Korea	38.084	127.025	MK893471	NA
MT584151	Calliphoridae	Calliphorinae	*Calliphora*	*C. vomitoria*	Denmark	55.674	12.566	MT584151	NA
KT936147	Calliphoridae	Calliphorinae	*Calliphora*	*C. chinghaiensis*	China	27.726	99.94	KT936147	NA
OX596080	Calliphoridae	Chrysomyinae	*Protophormia*	*P. terraenovae*	U.K.	55.25	-4.32	OX596080	NA
CM027232	Calliphoridae	Luciliinae	*Lucilia*	*L. sericata*	U.S.A.	NA	NA	CM027232	NA
MW255540	Calliphoridae	Luciliinae	*Lucilia*	*L. sericata*	Australia	-41.92	147.48	MW255540	NA
AJ422212	Calliphoridae	Luciliinae	*Lucilia*	*L. sericata*	U.K.	NA	NA	AJ422212	NA
JX913757	Calliphoridae	Luciliinae	*Lucilia*	*L. sericata*	U.S.A.	NA	NA	JX913757	NA
JX913754	Calliphoridae	Luciliinae	*Lucilia*	*L. sericata*	Australia	NA	NA	JX913754	NA
JX913756	Calliphoridae	Luciliinae	*Lucilia*	*L. sericata*	Australia	NA	NA	JX913756	NA
KT272854	Calliphoridae	Luciliinae	*Lucilia*	*L. sericata*	U.S.A.	NA	NA	KT272854	NA
JX913744	Calliphoridae	Luciliinae	*Lucilia*	*L. cuprina*	Australia	NA	NA	JX913744	NA
MW255538	Calliphoridae	Luciliinae	*Lucilia*	*L. cuprina*	Australia	-27.46	153.02	MW255538	NA
MW255539	Calliphoridae	Luciliinae	*Lucilia*	*L. cuprina*	Australia	-34.76	117.13	MW255539	NA
MW255536	Calliphoridae	Luciliinae	*Lucilia*	*L. cuprina*	Australia	-36.71	142.19	MW255536	NA
MW255537	Calliphoridae	Luciliinae	*Lucilia*	*L. cuprina*	Australia	-34.65	148.45	MW255537	NA
JX913749	Calliphoridae	Luciliinae	*Lucilia*	*L. cuprina*	Australia	NA	NA	JX913749	NA
KM657112	Calliphoridae	Luciliinae	*Lucilia*	*L. caesar*	U.K.	53.76	-2.23	KM657112	NA

KM657110	Calliphoridae	Luciliinae	*Lucilia*	*L. illustris*	U.K.	53.76	-2.23	KM657110	NA
KT272845	Calliphoridae	Luciliinae	*Lucilia*	*L. illustris*	U.S.A.	NA	NA	KT272845	NA
MT584139	Calliphoridae	Luciliinae	*Lucilia*	*L. illustris*	Denmark	NA	NA	MT584139	NA
MH540746	Calliphoridae	Luciliinae	*Lucilia*	*L. papuensis*	China	22.65	99.6	MH540746	NA
MT017717	Calliphoridae	Luciliinae	*Lucilia*	*L. papuensis*	China	35.4	116.57	MT017717	NA
JX913758	Calliphoridae	Luciliinae	*Lucilia*	*L. porphyrina*	Australia	NA	NA	JX913758	NA
MT872670	Calliphoridae	Luciliinae	*Lucilia*	*L. silvarum*	Denmark	NA	NA	MT872670	NA
MW592363	Calliphoridae	Luciliinae	*Lucilia*	*L. hainanensis*	NA	NA	NA	MW592363	NA
KT272780	Calliphoridae	Luciliinae	*Lucilia*	*L. coeruleiviridis*	U.S.A.	NA	NA	KT272780	NA
MW446947	Calliphoridae	Luciliinae	*Lucilia*	*L. shenyangensis*	China	39.8	116.46	MW446947	NA
JX913759	Calliphoridae	Luciliinae	*Hemipyrellia*	*H. ligurriens*	Australia	NA	NA	JX913759	NA
KT272778	Mesembrinellidae	Mesembrinellinae	*Mesembrinella*	*Mesembrinella* sp.*	Brazil	-23.529	-45.849	KT272778	NA
OP930888	Calliphoridae	Phumosinae	*Caiusa*	*Caiusa* sp.	China	NA	NA	OP930888	NA
OW121746	Calliphoridae	Rhiniinae	*Stomorhina*	*S. lunata*	U.K.	51.511	-1.112	OW121746	NA
OR497843	Calliphoridae	Rhiniinae	*Isomyia*	*I. nebulosa*	China	23.908	99.231	OR497843	NA
OW799239	Calliphoridae	Rhinophorinae	*Phyto*	*P. melanocephala*	U.K.	51.511	-1.112	OW799239	NA
OY757193	Calliphoridae	Rhinophorinae	*Melanophora*	*M. roralis*	U.K.	50.2	-4.949	OY757193	NA

Appendix 1													
	GenBank Accession of 13 Protein-coding Genes												
IDs	**ATP6**	**ATP8**	**COI**	**COII**	**COIII**	**CYTB**	**ND1**	**ND2**	**ND3**	**ND4**	**ND4L**	**ND5**	**ND6**
KEIB_DIP_02101	PQ438194	PQ438209	PQ428990	PQ438224	PQ438239	PQ438254	PQ438269	PQ438284	PQ438299	PQ438314	PQ438329	PQ438344	PQ438359
KEIB_DIP_02115	PQ438195	PQ438210	PQ428991	PQ438225	PQ438240	PQ438255	PQ438270	PQ438285	PQ438300	PQ438315	PQ438330	PQ438345	PQ438360
KEIB_DIP_02116	PQ438196	PQ438211	PQ428992	PQ438226	PQ438241	PQ438256	PQ438271	PQ438286	PQ438301	PQ438316	PQ438331	PQ438346	PQ438361
KEIB_DIP_02121	PQ438197	PQ438212	PQ428993	PQ438227	PQ438242	PQ438257	PQ438272	PQ438287	PQ438302	PQ438317	PQ438332	PQ438347	PQ438362
KEIB_DIP_02123	PQ438198	PQ438213	PQ428994	PQ438228	PQ438243	PQ438258	PQ438273	PQ438288	PQ438303	PQ438318	PQ438333	PQ438348	PQ438363
KEIB_DIP_02131	PQ438199	PQ438214	PQ428995	PQ438229	PQ438244	PQ438259	PQ438274	PQ438289	PQ438304	PQ438319	PQ438334	PQ438349	PQ438364
KEIB_DIP_02132	PQ438200	PQ438215	PQ428996	PQ438230	PQ438245	PQ438260	PQ438275	PQ438290	PQ438305	PQ438320	PQ438335	PQ438350	PQ438365
KEIB_DIP_02137	PQ438201	PQ438216	PQ428997	PQ438231	PQ438246	PQ438261	PQ438276	PQ438291	PQ438306	PQ438321	PQ438336	PQ438351	PQ438366
KEIB_DIP_01714	PQ438202	PQ438217	PQ428998	PQ438232	PQ438247	PQ438262	PQ438277	PQ438292	PQ438307	PQ438322	PQ438337	PQ438352	PQ438367
KEIB_DIP_02096	PQ438203	PQ438218	PQ428999	PQ438233	PQ438248	PQ438263	PQ438278	PQ438293	PQ438308	PQ438323	PQ438338	PQ438353	PQ438368
KEIB_DIP_02088	PQ438204	PQ438219	PQ429000	PQ438234	PQ438249	PQ438264	PQ438279	PQ438294	PQ438309	PQ438324	PQ438339	PQ438354	PQ438369
KEIB_DIP_02089	PQ438205	PQ438220	PQ429001	PQ438235	PQ438250	PQ438265	PQ438280	PQ438295	PQ438310	PQ438325	PQ438340	PQ438355	PQ438370
KEIB_DIP_02076	PQ438206	PQ438221	PQ429002	PQ438236	PQ438251	PQ438266	PQ438281	PQ438296	PQ438311	PQ438326	PQ438341	PQ438356	PQ438371
KEIB_DIP_02090	PQ438207	PQ438222	PQ429003	PQ438237	PQ438252	PQ438267	PQ438282	PQ438297	PQ438312	PQ438327	PQ438342	PQ438357	PQ438372
KEIB_DIP_02128	PQ438208	PQ438223	PQ429004	PQ438238	PQ438253	PQ438268	PQ438283	PQ438298	PQ438313	PQ438328	PQ438343	PQ438358	PQ438373
KEIB_DIP_02157	PV593855	PV593860	PV591018	PV593865	PV593870	PV593875	PV593880	PV593885	PV593890	PV593895	PV593900	PV593905	PV593910
KEIB_DIP_02194	PV593856	PV593861	PV591019	PV593866	PV593871	PV593876	PV593881	PV593886	PV593891	PV593896	PV593901	PV593906	PV593911

ARTICLES

ARTICLE 1

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Current Opinion in Insect Science

Blowfly genomics: current insights, knowledge gaps, and future perspectives

Drashti R Parmar[1], Nikolas P Johnston[2], James F Wallman[3,4] and Krzysztof Szpila[1]

Blowflies (Calliphoridae) form a diverse, species-rich group, yet publicly available genome assemblies are limited to only 16 species, despite recent genomic advances. This knowledge gap extends to mitogenomes and barcode databases, which mainly focus on medically and veterinary-important species. While blowfly phylogenetics has progressed, additional genome sequencing is crucial for various subfamilies, given their diverse life histories. This review presents a quantitative overview of available genetic information for blowflies, highlighting substantial gaps in public databases. DNA barcodes, mitogenomes, and genomes represent only 16.5% (342 species), ~3% (53 species), and < 1% (16 species) of known family diversity, respectively. While 183 genomics-related calliphorid BioProjects are recorded by NCBI, many subfamilies and genera have limited or no genomic representation, impacting studies on identification, systematics, phylogenetics, and evolution. We stress the urgent need for high-quality reference genomes and highlight target species representing all blowfly subfamilies to support a new era of rapid, low-cost genomic research.

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Introduction

The blowflies (Diptera: Calyptratae: Calliphoridae) comprise ~2075 species across 154 genera, according to the recent classification [1]. Despite accounting for 9% of calyptrate diversity and 1.3% of described Diptera, the true number of blowfly species is likely underestimated, as with most dipterans [2]. Calliphoridae belongs to the schizophora grade, a taxonomic group that has significantly impacted on shaping human civilization and advancing scientific studies [1,3]. Interestingly, despite their relative species diversity, as well as ecological and economic importance, the Calliphoridae family has suffered from a substantial gap in genomic knowledge. Compared to other well-studied dipteran families like Drosophilidae and Culicidae (~18% and ~4% of known species assembled, respectively), only ~1.6% of calliphorid species diversity is represented in available genome assemblies. This genomic representation is also lower than other comparably less speciose calyptrate dipteran families such as Polleniidae (~5.4%), Glossinidae (~25.8%), Anthomyiidae (~3.9%), and Hippoboscidae (~3.3%). The dearth of genomic information for most calliphorid taxa underscores the need to summarize existing genomic efforts and chart future priorities for genomic research in this group.

Calliphoridae are globally significant in medical, veterinary, and economic contexts, with many species acting as synanthropic scavengers and mammal parasites [3,4]. Despite recent progress in the higher-level phylogenetics of the Calyptratae and Oestroidea, intra-subfamily relationships remain understudied [1,5–8]. The classification and resolution of the calliphorid phylogeny have been contentious due to sparse taxon sampling and methodological discrepancies, leading to limited and conflicting hypotheses [1,4–15]. Despite advances in blowfly genomics, particularly with recent sequencing technologies, many entire calliphorid subfamilies lack targeted sequencing efforts. This is especially important as the constituent species of these subfamilies display taxonomically and behaviorally diverse life histories, including multiple origins of parasitism, parasitoidism, pollination, larval feeding habits, host choice, adaptive capacity, and myiasis.

Besides phylogenomics and systematics, new and cost-effective genomic tools could uncover genetic mechanisms behind the diverse behaviors and ecological, physiological, and adaptive traits of blowflies across different geographies, especially among morphologically similar species. Genomic comparison between species with

different trophic specialties could reveal coevolutionary dynamics in host specificity, detection, and immune evasion [16]. Currently, genomic knowledge of blowflies outside of medical, veterinary, and forensic contexts is limited. Expanding the diversity of available blowfly genomes using next-generation sequencing (NGS) technologies, such as long-read sequencing, is crucial for resolving phylogenetic controversies and understanding genetic bases of diverse life histories and evolutionary origins, as demonstrated in other insects [17]. This review aims to provide a quantitative overview of genetic information available for the Calliphoridae, highlighting available genomic resources and methodological approaches. We discuss disparities in genomic representation, highlighting an overemphasis on medically and veterinary important flies, and identifying a geographic bias in past research into this group. We stress the urgent need for high-quality reference genomes and highlight target species representing all blowfly subfamilies to support a new era of rapid, low-cost genomic research.

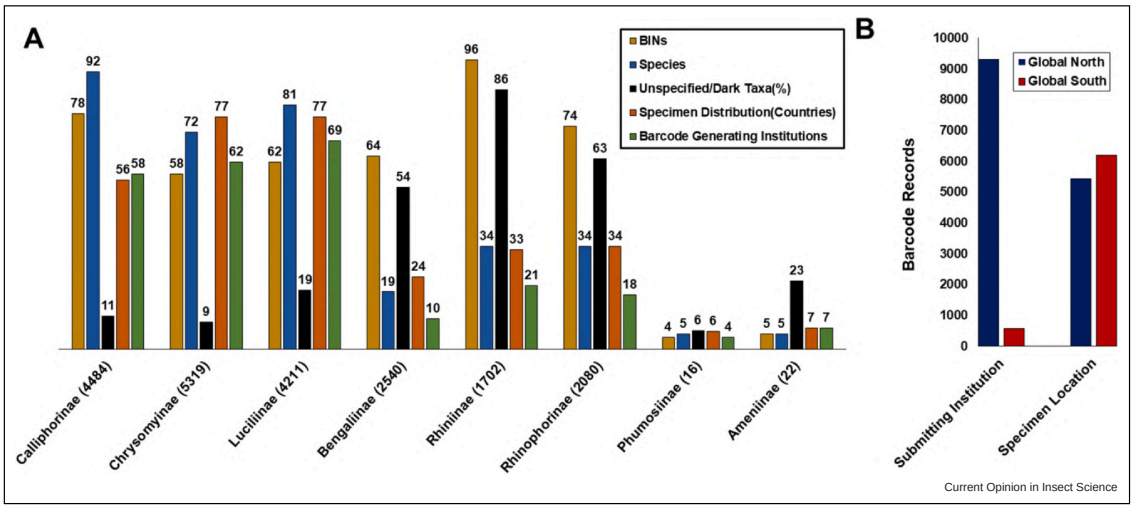
Genomic progress and key findings

Species identification and DNA barcoding

The species diversity within the family Calliphoridae underscores the importance of precise species identification, especially in the context of forensic entomology and veterinary science [18]. While traditional morphological identification is challenging [19], especially in distinguishing morphologically and behaviorally similar species [20], DNA barcoding, utilizing the 5′ end (~650bp) of the mitochondrial cytochrome oxidase 1 (COI) [18,21], solves this issue.

Along with GenBank [22], the Barcode of Life data system (BOLD) [23] is a primary database hosting barcode data [24]. As compared to similar-sized dipteran families like Culicidae (86 863 records), Psychodidae (239 614 records), Mycetophilidae (107 619 records), and Sciaridae (770 178 records), blowflies (21 941 records) have a relatively low number of barcodes on BOLD (August, 2024). From the 21 941 Calliphoridae records on BOLD, 583 barcode-compliance clusters with barcode index numbers (BINs) are formed. These BINs represent only 16.5% of described blowfly species, with 65.8% having designated species names, accounting for 342 species across 82 genera. Calliphorinae, Luciliinae, and Chrysomyinae remain the most heavily represented groups globally (Figure 1a). Surprisingly, around 24% of the barcode records lack a scientific name and therefore remain as 'dark' taxa (Figure 1a). The percentage of species barcoded compared to the known species diversity in blowflies (16.5%) lags behind Culicidae (45.7%), Psychodidae (17.9%), Mycetophilidae (44.9%), and Sciaridae (56.8%). This representation, alongside the bias towards veterinary and medically relevant groups in current blowfly barcode data, underscores the need for investment in an extensive and well-annotated barcode reference library. National initiatives under the

Figure 1



Calliphoridae DNA barcode data on BOLD (Aug, 2024). **(a)** DNA barcode distribution across calliphorid subfamilies, including deposited BINs (Barcode Index Numbers), number of species, unspecified/dark taxa, number of countries represented, and data deposition institutions. The number beside each subfamily in () represents the total barcode records on BOLD. **(b)** Calliphoridae DNA Barcode records on BOLD: the submitting institute vs specimen location across the Global North (e.g. Australia, Canada, Europe, United States) and Global South (e.g. Africa, Asia including China, Mexico, Middle East, South America).

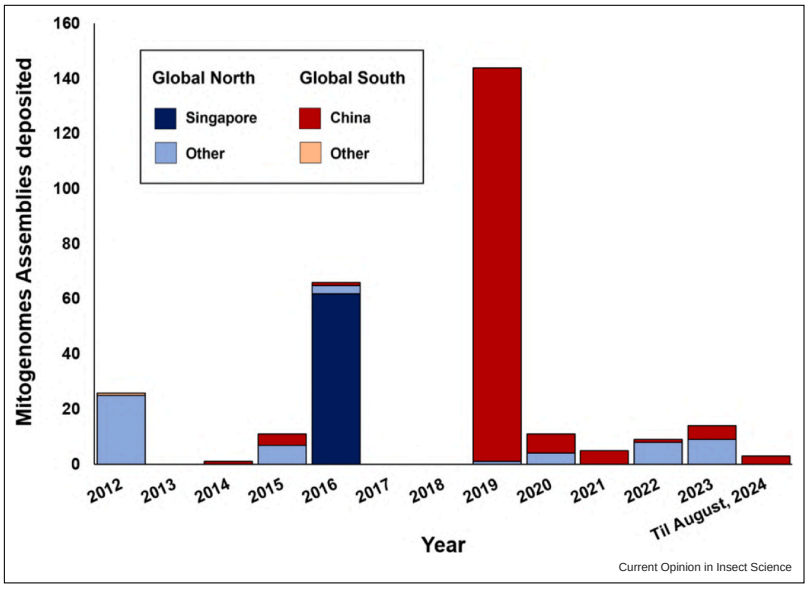
auspices of the International Barcode of Life have significantly expanded barcode libraries for native fauna [25,26]. However, the inclusion of blowfly taxa in these initiatives remains limited. Focused efforts within national initiatives to study local diversity and compare barcodes across their geographical distributions can potentially unveil shared [27], overlooked, and new species [28], supporting biodiversity inventories [29]. As such, generating comprehensive reference blowfly libraries at both national and global scales should be a priority.

Blowfly mitogenomes

Advanced and affordable NGS technologies have popularized mitochondrial genome analyses for in-depth phylogenetics and population studies [30]. Over the past two decades, complete mitochondrial sequences of Calliphoridae in public databases have increased exponentially (Figure 2), with 290 complete/near-complete mitogenomes representing 53 species, that is, ~3% of known taxa (August 2024, GenBank). These mitogenomes are primarily from economically and forensically important taxa, leaving other blowfly taxa poorly understood [5,6,31]. While these studies have provided insights into mitochondrial base composition, structure, codon usage, gene arrangement, and importance of third codon positions in protein-coding genes [6,32,33], the limited taxonomic coverage within the Calliphoridae restricts broader molecular and genomic explorations, particularly for comparative studies on ecological and evolutionary dynamics at the species level. Despite the considerable wealth of mitogenome resources within the Calliphoridae, additional sequencing efforts are needed, particularly in subfamilies with multiple origins of key blowfly behaviors such as parasitism or necrophagy. We recommend prioritizing broader taxonomic coverage within the blowfly family, including nonmodel flies, to reduce bias and expand the evolutionary scope of future studies.

Despite a substantial increase in blowfly molecular data in recent years, there exists a considerable geographic bias in the locations from which species are sequenced. Being the hub for leading genomic facilities, the Global North ('richer' globalized countries, e.g. Australia, Canada, Europe, Israel, Japan, New Zealand, Singapore, South Korea, United States) are far ahead towards the contribution of genomic data compared to the Global South ('poorer' developing countries, e.g. Africa, Asia including China, Mexico, Middle East, South America) [34]. Like other insect groups, blowfly sequencing efforts for both barcoding (Figure 1b) and complete genome assemblies (Figure 3a) are also evidently biased towards Global North countries. However, blowfly specimen distribution is skewed towards Global South countries (barcode data), representing > 53% of specified location records (Figure 1b). This uneven distribution highlights the disparity in sampling efforts and technological access across regions, which is responsible for current limitations in blowfly genetic information. This bias is inverted when we look at mitogenome assemblies,

Figure 2



Year-wise blowfly mitogenome assemblies in GenBank, deposited by major countries in the Global North and Global South.

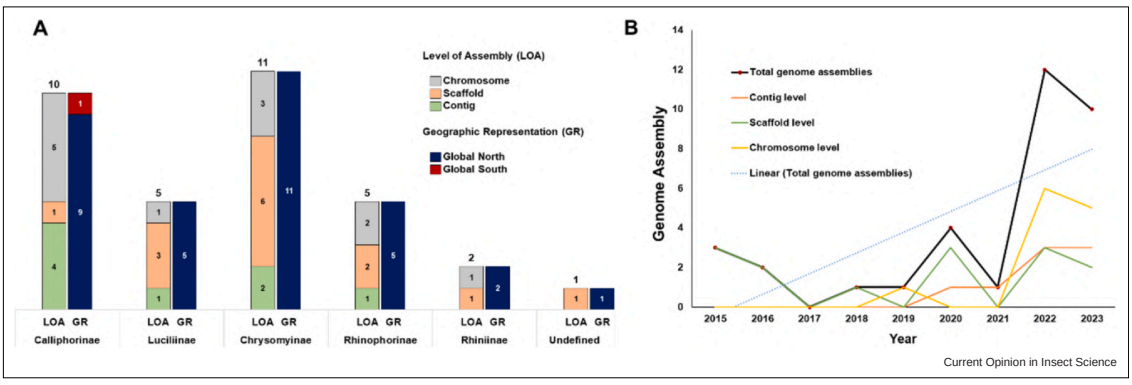
with the Global South (171) contributing more than the Global North (119), which could be attributed to the lower cost of mitogenome sequencing projects. It should, however, be noted that the contribution from the Global South is inflated by over 140 mitogenomes of *Chrysomya megacephala* (Fabricius) from a single location in China (Figure 2). Ensuring a wide coverage of blowfly taxa is crucial to avoid geographical bias, as limited sampling hinders robust conclusions about diversity, functional biology, habitats, life traits, and evolution. Implementing umbrella projects focusing on the regional and global biodiversity of calliphorid subfamilies can drive the generation of high-quality genome assemblies worldwide across a broader range of yet-to-be-sampled taxa. Therefore, we recommend conducting more studies with global geographic representation, especially in areas with minimal human disturbances.

Blowfly genomes

Currently, genome assemblies are available for 16 blowfly species (34 records), spanning 5 subfamilies and 13 genera, representing < 1% of the known family diversity. Among the available data, 12 species have chromosome-level assemblies, while the remaining 4 have scaffold-level assemblies (Figure 3a). Most of these genomes were sequenced in the past five years (Figure 3b), with most calliphorid genera still lacking genomic representation in public databases. The first chromosome-level reference assembly within the Calyptratae, *Calliphora grahami* (Aldrich) [35], was aimed at facilitating broader genomic research in blowflies. However, most available genome assemblies are focused primarily on medically and economically important blowfly species, such as veterinary pests *Lucilia sericata* (Meigen) [36] and *Lucilia cuprina* (Wiedemann) [37]. While both assemblies marked significant progress in understanding mechanisms behind sheep strike, the identification of protein secretions in *L. sericata*, in particular, is more critical, given its extensive application in maggot debridement therapy [36]. The New World screw-worm, *Cochliomyia hominivorax* (Coquerel), is known as a major livestock pest in South America and the Caribbean apart from being a public health threat, which was recently assembled in the United States [38,39]. Additionally, the genome sequences of several forensically important carrion breeding flies, including *Phormia regina* (Meigen), *Chrysomya rufifacies* (Macquart), *Calliphora vicina* (Robineau-Desvoidy), and *C. grahami*, have contributed to our understanding of their life cycle and ecological traits [35,40,41]. *Phyto melanocephala* (Meigen), *Paykullia maculata* (Fallén), and *Melanophora roralis* (Linnaeus) from the subfamily Rhinophorinae were also sequenced for insights into parasitoid evolution and to support future comparative genomic studies [42]. While advancing technologies and bioinformatic tools have provided opportunities to capitalize on existing molecular resources [43], expanding genomic resources for blowflies with wider taxonomic coverage beyond model groups would enable broader phylogenetic comparisons and the assessment of family-wide genetic mechanisms while also offering deeper insights into the evolutionary processes at both species and population levels [16].

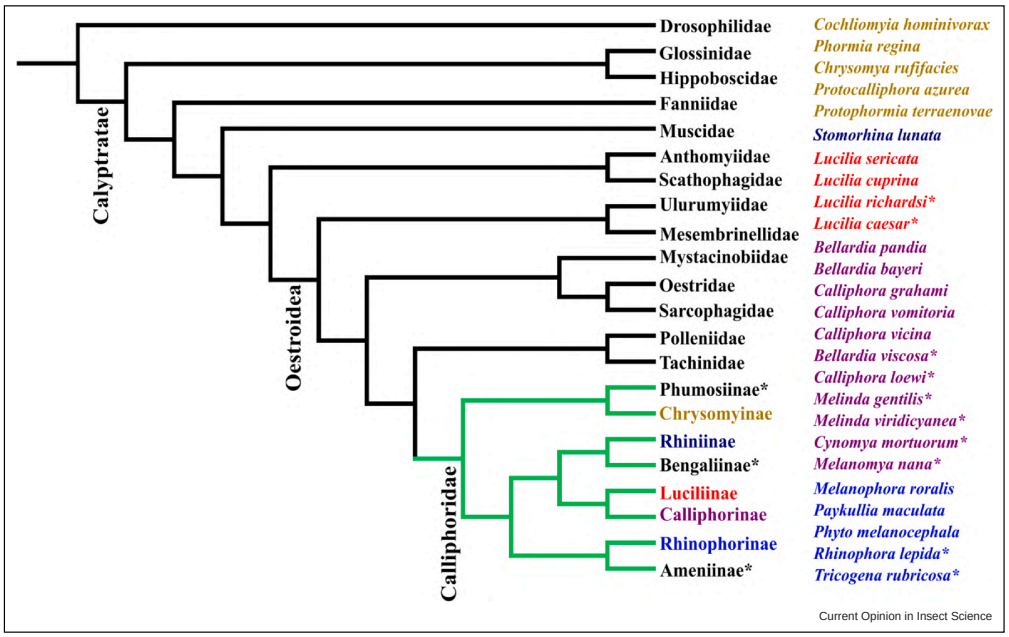
In the context of the most recent phylogenetic hypothesis of calliphorid classification (Figure 4, [1]), the subfamilies Phumosiinae, Bengaliinae, and Ameniinae lack representation with whole genome sequences. Notably, *Amenia imperialis* (Robineau-Desvoidy) exhibits parasitic lifestyles (e.g. snail parasite) and, like Phumosiinae, has unique reproductive strategies (unilarviparity), unlike most other calliphorid subfamilies (oviparity) [11], contributing to their high adaptability to extreme habitats

Figure 3



Complete genome assemblies in Calliphoridae: **(a)** Types of blowfly assemblies contributed from the Global North (e.g. Australia, Europe, United States, United Kingdom) and Global South (e.g. China) separated by subfamily. For each subfamily, Level of Assembly (LOA) and Geographic Representation (GR) are shown. **(b)** Yearly deposited data on public platforms, showing linear progress over the years in assembly efforts.

Figure 4



Phylogeny of Oestroidea as per Yan et al. [1] (Transcriptomic data). The classification of Calliphoridae follows the suggestions by Yan et al. [1], marked with green colored clade. Calliphorid subfamilies marked with (*) represent no reference genome assemblies to date and therefore of future targets. Species besides the tree represent either completed or submitted as biosamples/in progress (*) reference genome assemblies in the Darwin Tree of Life Project, Wellcome Sanger Institute. Colors of the species corresponds to calliphorid subfamilies on the tree.

and environmental gradients. Bengaliinae and Rhiniinae share parasitic behavior [1], suggesting a synchronized evolution trajectory within Calliphoridae [11]. Despite these insights, detailed studies on their life cycles and larval morphology are hindered by lack of genomic data.

Species in the genus *Chrysomya* (Robineau-Desvoidy) display high adaptability in different environmental conditions, contributing to their invasive traits across habitats [44]. For example, *Chrysomya albiceps* (Wiedemann), *C*hrysomya *putoria* (Wiedemann), *Ch. megacephala*, and *Ch. rufifacies* have been displacing native blowflies, such as *Cochliomyia macellaria* (Fabricius) in the Americas [44,45]. Genome-wide studies could provide vital information on invasive mechanisms affecting native species [46]. *Calliphora hilli* (Patton), *Calliphora stygia* (Fabricius), and *C. vicina* are invasive in New Zealand and have been studied to investigate the extent of gene flow, hybridization, and population structure [47]. However, with the exception of *Ch. rufifacies* and *C. vicina*, genome-wide studies remain scarce, limiting our understanding of the impact of invasive species on native fauna, carrion ecology, population structure, gene flow, genetic bottlenecks, and changes in selection pressures [47]. Even commonly occurring blowflies in their respective geographies, which are of forensic, medical, and veterinary importance, such as *Chrysomya bezziana* (Villeneuve), *Chrysomya marginalis* (Wiedemann), *Lucilia ampullacea* (Villeneuve), *Lucilia illustris* (Meigen), *Lucilia silvarum* (Meigen), and the above listed *Chrysomya* species lack whole genome assemblies to date. Agriculturally and forensically important Australian endemic *Calliphora augur* (Fabricius) and *Calliphora dubia* (Macquart) cause significant economic losses due to sheep myiasis [48]. The fluctuating weather patterns strongly influence sheep strike incidence [49,50], yet no genome-scale data exist for these species. Similarly, the Australian endemic Aphyssurinae (now included in Calliphorinae) also lacks genomic data. Genome-wide studies on such endemic/isolated blowflies could provide valuable insights into their dispersal capabilities, adaptive potential, population structure, disease incidence prediction, and associated management actions. Comprehensive knowledge of genetic mechanisms would shed light on these less-explored species groups and their biology, ecology, and species interactions under current and future climate conditions.

As of August 2024, the National Center for Biotechnology Information (NCBI) recorded 183 BioProjects on calliphorid

genomics across five subfamilies: Calliphorinae, Luciliinae, Chrysomyinae, Rhiniinae, and Rhinophorinae. These resources have enabled entomologists to generate extensive functional-genomic data from blowflies used in studies, including sex determination, transcriptomics, immunogenetics, microbial ecology, nutrition, and insecticide resistance (as summarized in Ref. [51]). However, many sequencing projects are not registered with the NCBI until publication, and there are likely additional genome projects underway for Calliphoridae. In addition to the extensive NCBI databases, initiatives like i5k [52], GigaDB (http://gigadb.org), and Insectbase [53] have significantly contributed to generating and/or housing blowfly genomic resources. The Wellcome Sanger Institute in the United Kingdom has also initiated genome assembly projects for several blowfly species, including *Bellardia pandia* (Walker), *Protocalliphora azurea* (Fallén), *Calliphora vomitoria* (Linnaeus), *C. vicina*, and *Stomorhina lunata* (Fabricius).

In addition to studying the genetic content of blowfly genomes, it can also be valuable to study the number and arrangement of chromosomes, that is, the karyotype, which can provide insights into chromosomal rearrangements, the distribution of sex chromosome systems, genetic exchange, and speciation mechanisms [54,55]. In blowflies, karyotyping studies have provided insights into conserved chromosomal gene content (Muller elements), structural mutations, variation in sex chromosome size and morphology, population divergence, and distinct evolutionary rates among closely related blowfly species [41,56–58]. Frequent sex chromosome transitions, with the presence of both homomorphic and heteromorphic sex chromosomes, highlight the influence of sex-specific selective pressures on sex chromosome evolution in blowflies [41,56,58]. Despite these findings, sex chromosome evolution and karyotype studies in blowflies remain limited, with data available for only ~90 calliphorid species (https://colecoguy.github.io/karyotypes/) [55]. Expanding comparative genomic studies with chromosomal-level genome assemblies across a broader taxonomic range would reveal large-scale phylogenetic patterns on the distribution of reproductive systems, karyotype evolution, and sex determination mechanisms, aiding the identification of key species for future genome assemblies advancing genome biology research [55].

Blowfly systematics and phylogenomics

Morphological evidence supporting calliphorid phylogenetics has historically been challenging due in part to convergent evolutionary traits within the Oestroidea [4,8]. This instability has resulted in the Calliphoridae being resolved as nonmonophyletic, based on ground-plan apomorphies [3,9,10,59]. Despite significant contributions made by Knut Rognes in blowfly systematics and phylogenetics, a limited number of detailed morphological studies have restricted morphological character sets (often homoplastic) for inferring blowfly phylogeny [9,14]. However, blowfly phylogenetics has been greatly benefiting from modern integrative studies that combine morphological and molecular data, gradually resolving taxonomic ambiguities and contributing to key findings related to adaptive evolution, cross-species hybridization, divergence, and phylogenetic relationships within the group [4,8,11,13,14,60,61].

Different hypotheses place Calliphoridae variously within the broader Oestroidea: 1) as sister to Sarcophagidae (morphological [10] and molecular data [5,6,8,62]); 2) paraphyletic at the base of Tachinidae and sister to the rest of Oestroidea (morphological [63] and molecular data [4,64]); 3) closer to Oestridae and Rhinophorinae/Rhinophoridae (morphological data [9,65]); 4) sister to Tachinidae (molecular data [14]); 5) sister to Tachinidae+Polleniidae (molecular data [62]); 6) nested within a monophyletic lineage with Sarcophagidae and Tachinidae, sister to Oestridae (*s.lat*) (morphological data [3,66]); or together with Mystacinobiidae, as a sister to other Oestroidea (molecular data [4]) or 7) sister to Rhiniinae/Rhiniidae+Tachinidae or Rhiniinae/Rhiniidae together with a clade consisting Oestridae+Rhinophorinae/Rhinophoridae+Tachinidae (molecular data [4,13]).

Despite over two decades of progress in molecular phylogenetics, the evolutionary relationships between the key subfamilies of the Calliphoridae remain poorly resolved, and the monophyly of the family itself has been contested, with studies resolving both a monophyletic [1,3,6,10,65] and a para/polyphyletic Calliphoridae [4,5,8,9,11,14,59,67]. The major contributing factors to the difficulty in accurately resolving the Calliphoridae are incomplete taxon sampling at the subfamily level and the inadequate identification and screening of orthologous loci across taxa [4,7,8,11,68]. One particular subfamilial relationship difficult to resolve is of the monophyletic Rhiniinae/Rhiniidae (either as a sister to traditional calliphorids with independent family rank [4,13] or as a subfamily of the Calliphoridae and sister to Bengaliinae [1,11]). Despite the complexity of this group, molecular data, particularly from large-scale phylogenomic studies, have provided enough evidence to revise the taxonomy of some major groupings. For instance, the raising of Polleniinae to Polleniidae [1,11,15] contradicts its placement within Calliphoridae *s.str.* as a subfamily [4,11–13,15]. Similarly, Bengaliinae have been suggested to be a subfamily closely related to Rhiniinae/Rhiniidae [1,15].

Blowfly phylogenomic studies have surged recently, presenting several comprehensive molecular phylogenies based on multilocus datasets. Kutty et al. suggested a paraphyletic Calliphoridae, based on mitochondrial and nuclear genes, encompassing most traditional calliphorid subfamilies [4]. However, the placement of Helicoboscinae, Rhiniinae/Rhiniidae, and

Rhinophorinae/Rhinophoridae conflicted as they were clustering alongside Tachinidae, Mesembrinellidae, and Polleniidae [4]. Molecular systematics of 10 calliphorid subfamilies (including Polleniidae and Mesembrinellidae with subfamily status), based on mitochondrial and nuclear data, placed Rhiniinae/Rhiniidae as sister to Bengaliinae, Toxotarsinae to Calliphorinae, and Helicoboscinae closer to Calliphorinae [11]. Mitogenomic approaches also addressed ambiguities, although limited to only three calliphorid subfamilies, and therefore lacked conclusive insights [12,31]. Divergence estimates across Oestroidea placed Calliphoridae's origin at ~29 million years ago and supported its paraphyly based on mitochondrial and nuclear data [14]. Transcriptomic data also recovered Calliphoridae as paraphyletic [7]. Analysis of protein-coding ultra-conserved elements (UCEs) resolved similar relationships, with Rhinophorinae/Rhinophoridae sister to traditional Calliphoridae, and the Polleniidae excluded from the Calliphoridae [15]. The most recent phylogenetic hypothesis by Yan et al. focused exclusively on resolving controversial relationships within the Calliphoridae using a phylogenomic dataset and redefined the family as a more inclusive monophylum, thereby addressing a long-standing debate within this group [1].

As a result of the recent progress in blowfly systematics, several taxonomic revisions have been completed, including reclassifying the subfamilies of the Calliphoridae (Figure 4), incorporating Aphyssurinae, Melanomyinae, and Toxotarsinae [1]. Ameniinae now includes Helicoboscinae, and Bengaliinae is expanded to include Auchmeromyiinae [1]. Additionally, Rhiniidae [4] and Rhinophoridae are now treated as calliphorid subfamilies instead of separate families [1,69], with Mesembrinellidae and Polleniidae excluded as calliphorid subfamilies under revised classifications [1,8,70].

Advances in genomic technologies

Technological advancements in sequencing technologies over the past two decades, including high-throughput short-read sequencing, followed by long-read sequencing, have enabled the generation of contiguous and high-quality blowfly (and other insect) genome assemblies [17]. NGS technologies, coupled with genome reduction methods, are the most common approach for generating nuclear phylogenomic datasets for blowflies. These approaches can include, but are not limited to, anchored hybrid enrichment [68], miRNA [64], and highly multiplexed amplicon-based phylogenomics [71], all of which have been applied successfully to dipteran phylogenetics. To date, the family Calliphoridae have been analyzed using targeted enrichment of UCE probes [15], albeit with limited taxon sampling and with transcriptomics, providing comprehensive insights into blowfly phylogenetics and evolution [1,7]. Future research could further leverage targeted capture approaches with taxon-specific probes [72] to elucidate conserved genes, their rates of evolution, selective pressures, and the phylogenetic framework of contentious blowfly clades, spanning from subfamily to species level.

Challenges, future directions, and conclusions

Despite significant progress in blowfly genomics, there are still challenges to address with limitations of genomic and transcriptomics data, sparse taxon sampling, and integration of multiomics approaches, which are crucial to fully resolve the evolutionary history of the Calliphoridae. Obtaining high-quality genome assemblies for rare and endemic blowfly species is hindered by factors such as genome size, repetitive regions, computational demand, and difficulties in specimen collection. A 'total evidence' approach, combining broader taxon and gene sampling with additional character systems like morphology, physiology, and genome architecture, can accurately place challenging taxa in phylogenetic tree. However, careful consideration of biases with sampling, assembly methods, and genome quality is crucial before designing such experiments. With advancing technologies like RNAi and CRISPR/Cas9, genome-wide studies in blowflies have identified novel drug targets and biological control strategies to minimize the incidence of flystrike by the species *L. cuprina* and *C. hominivorax* [73–75]. Similar genomic studies on other veterinary-important blowfly species such as *C. albifrontalis* (Malloch), *C. augur*, *C. dubia*, *C. stygia*, and *Ch. megacephala* could also identify new targets, thereby improving pest management strategies. Furthermore, genome-scale studies could also aid in the characterization of therapeutic compounds from blowflies, such as those identified from *L. sericata* [76] and *Sarconesia magellanica* (Le Guillou) [77]. Similar to findings in *Anopheles* (Meigen) [78], *Drosophila* (Fallén) [79], Diptera, and Hymenoptera [80], advances in sequencing technologies and genome-wide comparative studies could offer deeper insights about blowfly biology, particularly in areas like drug resistance and host attraction [51].

In summary, the future of blowfly research looks promising in the wake of technological advancements. While substantial progress has been made since the pioneering work of Hennig, it is clear that much work has to be done to resolve controversial lineages within the Calliphoridae. Initial genomic explorations were focused on species with perceived economic importance, but now studies can efficiently encompass a broader array of ecologically significant blowfly taxa, aiming to bridge gaps of uneven genomic sampling by leveraging cost-effective technologies. The current understanding of blowfly subfamily relationships is fragmented, demanding thorough investigation for diverse applications

in medicine, veterinary science, and forensic science. The highest priority targets for new genomic assemblies should be those calliphorid subfamilies for which no genomic data are available (identified in Figure 4). Looking forward, as entomological research evolves with the rapid acceleration of data generation in the genomic era, effective data sharing and management, global collaborations, dedicated online platforms, and innovative automated technologies will be even more important.

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CRediT authorship contribution statement

Drashti R Parmar: Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing. **Nikolas P Johnston**: Writing – original draft, Writing – review & editing. **James F Wallman**: Writing – review & editing. **Krzysztof Szpila**: Funding acquisition, Supervision, Writing – review & editing.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT to improve readability. After using this tool/service, the authors carefully reviewed and edited the content as needed and took full responsibility for the content of the publication.

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- •• of outstanding interest

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ARTICLE 2

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Large-scale comparative analysis of blowfly mitogenomes, with an emphasis on Calliphorinae (Diptera: Calliphoridae), provides evolutionary insights into mitochondrial DNA structure, gene rearrangements, and inter- and intra-subfamilial relationships among blowflies

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ABSTRACT

Blowflies (Calliphoridae) represent a species-rich group, comprising 9 % of calyptrate diversity and 1.3 % of all described Diptera. The blowfly subfamily Calliphorinae, is known for its species of medical, veterinary and forensic importance, which has led to a skew toward these species in publicly available mitochondrial genome data. This skew leaves a significant proportion of this subfamily unrepresented and has resulted in poorly understood inter- and intra-subfamilial relationships, due to restricted taxon sampling. To address this, we assembled mitogenomes for 63 Calliphorinae species across 14 genera and a further 15 species from all remaining calliphorid subfamilies, representing the most comprehensive mitogenomic dataset for blowflies to date. Comparative analysis of mitochondrial DNA revealed structural and functional variations in gene order, base composition, codon usage, evolutionary rates and gene rearrangements across blowfly lineages, including a major rearrangement in *Calliphora varifrons*. Phylogenetic analysis of 13 mitochondrial protein-coding genes strongly supported the monophyly of Calliphoridae and its subfamilies. Clades Chrysomyinae (Calliphorinae+Luciliinae) and Rhiniinae+Bengaliinae are well-supported, with former subfamilies Melanomyinae and Toxotarsinae nested within Calliphorinae. Ameniinae was paraphyletic in most analyses, with variable placement of *Eurychaeta palpalis*. Genera *Calliphora* and *Onesia* are non-monophyletic, and the synonymisation of *Calliphora* with *Cynomya*, *Cyanus* and *Cynomyiomima* is suggested.

1. Introduction

Mitochondrial genomes (mitogenomes) are among the most extensively studied genomic systems and have emerged as highly valuable markers for taxonomic, phylogenetic and evolutionary analyses in insects, including microevolutionary processes and genetic variability [1–7]. Their compact, conserved gene structure, high mutation rates, and maternal inheritance make them particularly informative for resolving evolutionary relationships across taxonomic ranks [2,8]. Common metrics of compositional heterogeneity such as AT/GC skews have been linked to accelerated gene rearrangement rates and can reflect lineage-specific evolutionary dynamics, including selection pressures, metabolic needs, and genome structural constraints [9–11].

Phylogenetically informative events like mitochondrial gene rearrangements, although rare and random, have been recorded across many insect orders [2,12,13] such as: Phthiraptera [14], Thysanoptera [15,16], Hymenoptera [17], Hemiptera [11], Psocoptera [18] and Trichoptera [19], shedding light on deep evolutionary patterns across lineages [2]. In Diptera, while major rearrangements (involving Protein-Coding Genes (PCGs) and rRNAs) are uncommon, with limited instances reported in Ceratopogonidae [20] and Tachinidae [5], tRNA duplications are more frequent and have been reported in several calliphorid genera, including *Chrysomya* Robineau-Desvoidy, *Phormia* Robineau-Desvoidy, and *Calliphora* Robineau-Desvoidy [21–25].

In addition to resolving deep phylogenetic relationships [2,26–28], recent comparative mitogenomic studies in Diptera have also elucidated patterns of nucleotide composition, substitution rates, selective pressures and gene rearrangements, providing insights into mitochondrial

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genome evolution and aiding taxonomic revisions [3,5–7]. However, many fly groups remain underrepresented in mitogenomic databases, limiting our understanding of their evolutionary history.

Blowflies (Diptera: Calyptratae: Calliphoridae) are a species-rich family, comprising over 2000 described species across 154 genera [29,30], however, the actual diversity is likely underestimated, similar to many Dipteran groups [31]. The Calliphoridae are ecologically and economically important with roles in decomposition, pollination and as agents of myiasis in humans and animals [32–35]. Yet, their classification and phylogenetic resolution remains contentious, hindered by inconsistent taxon sampling and conflicting morphological and molecular hypotheses at both the familial and subfamilial level. The monophyly of Calliphoridae and several subfamilies, including Calliphorinae remains unresolved, with historically problematic genera (e.g., *Calliphora*, *Cynomya* Robineau-Desvoidy, *Cyanus* Hall, and *Cynomyiomima* Rohdendorf) often recovered in discordant phylogenetic positions [25,30,34,36–44].

Calliphorinae, once considered a clearly defined subfamily based on morphology [45], is now part of a more complex classification comprising eight subfamilies. With consistently disputed monophyly, molecular studies often place it sister to Luciliinae [30,34,36,41,44,46], whereas morphological data align it more closely with Chrysomyinae [40]. Further confusion surrounds Toxotarsinae and Aphyssurinae: the former is often molecularly allied with Calliphorinae [30,41,44], but not morphologically [47], while the latter shows conflicting affinities with Calliphorinae, Ameniinae or Phumosiinae depending on the data source [30,43]. Recent molecular studies have prompted substantial taxonomic revisions, including the reclassification of several subfamilies (e.g., merging Aphyssurinae and Melanomyinae into Calliphorinae) and exclusion of families such as Polleniidae and Mesembrinellidae from the Calliphoridae [30,38,48,49].

Despite growing interest in blowfly systematics, the family as a whole remains underexplored at the mitogenomic level, with many entire calliphorid subfamilies still lacking complete mitogenomes for any representative taxa, which restricts comprehensive molecular and mitogenomic studies within this group. Fewer than 3 % of described blowfly species are currently represented by complete or near-complete mitogenomes on public databases (>290 sequences representing 53 species) [50]. These data are disproportionately biased toward economically and forensically important groups, leaving substantial genomic gaps across subfamilies. [24,25,36,42,51]. Addressing these gaps is particularly important to capture the full spectrum of taxonomic and behavioral diversity that is present across blowfly subfamilies, including multiple independent origins of parasitism, parasitoidism, diverse pollination strategies, larval feeding behaviors and host preferences [34,39,43,46].

This dearth of available blowfly mitogenomic data is further emphasized within the blowfly subfamily Calliphorinae, with only 25 complete or near-complete mitogenomes from 16 species (7 genera) publicly available as of March 2025 (NCBI, GenBank)—64 % of which are from forensically important genus, *Calliphora*. To date, only 28 species across 11 Calliphorinae genera have been examined with any molecular data, leaving large portions of the subfamily molecularly untested (Table 1). As expected, the flow on effect of this lack of available data is incongruencies between phylogenetic studies with some (including the most recent) supporting a monophyletic Calliphorinae [40,42,46,52,53], and alternate studies resolving the group as para/poly-phyletic [30,34,36,44]. Even the monophyly of widely distributed genera such as *Calliphora* and its placement within broader Calliphorinae itself remains uncertain [36,46,52], in part due to limited taxon sampling across this genus (Table 1).

Progress in resolving these issues requires methods that leverage a greater variety of material, particularly as many species are not readily available nor able to be collected with baits or traps. To this end, genome skimming, a low-coverage whole-genome sequencing approach has proven efficient and cost-effective for assembling mitogenomes from

Table 1
Summary of previous molecular phylogenetic studies on Calliphorinae and their methodologies, in ascending order of their publication.

Genera	Constituent species	Methodology	Relevant citations
Calliphora *Cynomya* *Onesia* *Bellardia* *Calliphora* *Cynomya* *Melinda* *Sarconesia*	*C. dubia* *C. quadrimaculata* *C. stygia* *C. vicina* *C. vomitoria* *C. cadaverina* *C. mortuorum* *O. tibialis* *B. vulgaris* *C. vomitoria* *C. mortuorum* *M. viridicyanea* *S. chlorogaster* *S. versicolor*	Mitochondrial (COI, COII) + nuclear (28S) data Mitochondrial (12S, 16S, COI, CYTB) + nuclear (18S, 28S, CAD, Ef1α) data	[52] [34]
Calliphora *Cynomya* *Onesia* *Calliphora* *Sarconesia* *Calliphora* *Calliphora* *Cynomya* *Bellardia* *Melinda* *Onesia* *Sarconesia* *Calliphora* *Calliphora* *Calliphora* *Sarconesia* *Aphyssura* *Calliphora* *Onesia* *Melanomya* *Melinda* *Sarconesia* *Toxotarsus* *Calliphora* *Bellardia* *Calliphora* *Melanomya* *Melinda* *Pericallimyia* *Sarconesia* *Aphyssura* *Calliphora* *Melinda* *Polleniopsis* *Sarconesia* *Calliphora*	*C. dubia* *C. quadrimaculata* *C. stygia* *C. vicina* *C. vomitoria* *C. cadaverina* *C. mortuorum* *O. tibialis* *C. croceipalpis* *C. vicina* *C. vomitoria* *S. chlorogaster* *C. vicina* *C. vomitoria* *C. cadaverina* *B. vulgaris* *M. viridicyanea* *O. tibialis* *S. chlorogaster* *S. versicolor* *C. grahami* *C. vicina* *C. vicina* *C. croceipalpis* *C. vicina* *S. chlorogaster* *Aphyssura* sp. *C. vicina* *C. vomitoria* *O. tibialis* *M. bicolor/abdominalis* *Melinda* sp. *Sarconesia* sp. *S. chlorogaster* *T. ambrosiana* *C. vomitoria* *B. viarum* *Calliphora* sp.n. 1 *M. nana* *M. viridicyanea* *Pericallimyia io* *S. versicolor* *Aphyssura* sp. *C. vomitoria* *M. viridicyanea* *Polleniopsis* sp. *S. magellanica* *C. calliphoroides* as *"Triceratopyga"* *C. chinghaiensis* *C. grahami* as *'Aldrichina'*	Mitochondrial (COI, 16S) + nuclear (ITS2, 28S) data Mitochondrial data (Complete mitogenome) Mitochondrial (COI) + nuclear (28S, CAD, Ef1α) data Mitochondrial data (Complete mitogenome) Mitochondrial data (Complete mitogenome) Mitochondrial (COI, COII, 16S rDNA) + nuclear (ITS2, 28S rDNA) data Mitochondrial (16S) + nuclear (28S, CAD) data Phylotranscriptome (Single-copy protein-coding genes) data Ultra-Conserved Elements (UCE) loci Single-copy protein-coding gene data Mitochondrial data (13PCGs + 2 rRNAs)	[38] [25] [41] [42] [51] [39] [43] [37] [44] [30] [36]

(*continued on next page*)

Table 1 (*continued*)

Genera	Constituent species	Methodology	Relevant citations
	C. nigribarbis *C. sinensis* *C. uralensis* *C. vicina* *C. vomitoria*		
Polleniopsis *Calliphora* *Melinda* *Polleniopsis*	*P. mongolica* *C. vomitoria* *M. viridicyanea* *Polleniopsis* sp.	Single-copy protein-coding gene data	[116]

poorly preserved, low- quality or degraded DNA, including museum specimens [54–56]. This technique bypasses the need for species-specific optimization or prior genomic knowledge, making it ideal for large-scale mitogenomic surveys in poorly studied taxa [57,58].

Here, we leverage genome skimming techniques to generate 96 complete or near-complete mitogenomes across 78 blowfly species (28 genera), with a focus on Calliphorinae (63 species, 14 genera). Our study represents the largest mitogenomic survey of Calliphoridae to date and provides both, the first blowfly comparative analysis and a new comprehensive molecular phylogenetic hypothesis for the Calliphorinae based on mitochondrial data. Specifically, we aim to:

1. Characterize complete mitogenomes and perform comparative analyses of genomic structure, base composition, substitution rates, and gene rearrangements to understand mitogenome evolution across blowfly lineages.
2. Examine compositional biases, substitutional variations, and selection pressures to assess lineage-specific evolutionary adaptations.
3. Construct a robust phylogeny of Calliphoridae, with emphasis on resolving subfamily and genus-level relationhips within Calliphorinae.

By addressing these objectives, our study aims to resolve long-standing taxonomic ambiguities, provide a deeper understanding of blowfly evolutionary relationships and mitogenome evolution, and broaden the available mitogenomic database for this ecologically and economically important insect family.

2. Material and methods

2.1. Taxon sampling

Specimens were obtained through both field surveys and from museum collections (Fig. S1, Table S1). In some cases, multiple representatives of the same species from different locations were also sampled to assess potential genetic variation between geographically isolated populations. The specimens were digitally documented using an M205 C Leica Stereomicroscope with an integrated high-resolution Leica DFC495 digital camera and associated software (Leica Application Suite 4.4.0). 1–2 hind legs from pinned specimens and a part of thorax from ethanol preserved specimens were used for DNA extraction. Specimen vouchers are currently housed in the Department of Ecology and Biogeography, Nicolaus Copernicus University, Poland.

A total of 96 complete or near-complete mitogenomes were assembled in this study. For mitogenome characterization, we analyzed 114 specimens, representing 89 species from 35 genera, including both newly generated data and reference sequences from NCBI. For phylogenetic inference, we analyzed 107 mitogenomes (including reference sequences from NCBI), representing 84 species from 33 genera, with Ulurumyiidae and Mesembrinellidae used as outgroups (Tables S1, S2). The nomenclature and classification used in this study follows Rognes [32] and Yan et al. [30].

2.2. DNA extraction, library preparation and sequencing

Total genomic DNA was extracted from 1 to 2 legs of pinned specimens or from thorax muscle tissue of ethanol-preserved specimens using DNeasy Blood & Tissue Kit (Qiagen Valencia, CA, USA) and stored at −20 °C for further analysis. DNA concentration was quantified by Qubit 3.0 fluorometer using a dsDNA High Sensitivity Assay Kit (Life Technologies, Inc., Carlsbad, CA, USA). The quality and integrity of DNA extracts were analyzed using gel electrophoresis on 0.5 % agarose gels (Biotium, Darmstadt, Germany). Genome skimming techniques [55] were then used to construct genomic libraries, as described in Johnston et al. [27], with the modification outlined below. Input DNA was mechanically sheared using a Covaris M220 Focused-ultrasonicator (Covaris, Brighton, UK), which was then used to construct two pooled NEBNext Ultra II DNA libraries (New England BioLabs Inc., Ipswich, MA, USA) with an insert size of 200 base pairs (bps). Libraries were indexed using unique NEBNext Multiplex Oligos for Illumina (New England BioLabs Inc., Ipswich, MA, USA). The library quality and size distribution were verified on Agilent 2100 Bioanalyser using a high sensitivity DNA chip (Agilent Technologies, Inc., Santa Clara, CA, USA). Pippin Prep (Sage Science, Beverly, MA, USA) was used for size selection of 170–400 bp and to remove library fragments containing only adapters. Paired-end sequencing with a read length of 100 bp was performed on an Illumina NovaSeq 6000 platform at Macrogen Europe (Amsterdam, The Netherlands). An average of ~1 Gb of clean data was obtained from each library after trimming using Trimmomatic [59].

2.3. Mitogenome assembly and annotation

De novo assemblies of raw read data were performed using MitoZ [60] with the MegaHit assembler [61]. More than 80 % of the target mitogenomes were accurately assembled by MitoZ. To retrieve the remaining mitogenomes, we tested three alternate assembly pipelines on MitoFinder [62], as implemented recently with calyptrates [27]: (1) metaSPADES [63], (2) MegaHit [64], and (3) IDBA-UD [65]. *Lucilia cuprina* Wiedemann (GenBank accession no. JX913744.1 [25], Luciliinae: Calliphoridae) was used as a reference for the assembly and annotation in MitoFinder. Using a combination of these four approaches, we were able to successfully assemble mitogenomes for our entire dataset. The length and accuracy of the assemblies were estimated in comparison to published mitogenomes of closely related species in NCBI.

The 22 tRNA genes were annotated using the default annotator, MiTFi [66] in MitoZ and ARWEN [67] in MitoFinder, with their secondary structures predicted by the tRNAscan-SE 2.0 Search Server (https://trna.ucsc.edu/tRNAscan-SE/) [68]. The protein coding genes (PCGs) and rRNAs were identified using tblastn [69] and MITOS2 [70], respectively. Tandem Repeats Finder v 4.10.0 [71] was used to identify the long non-coding control region (putative A + T-rich region). Partial or missing genes were annotated using MITOS2 [70] within the Galaxy online interface ([72]; https://usegalaxy.eu/). MITOS2 was also used to annotate six mitogenome assemblies of Calliphoridae extracted from the National Centre for Biotechnology Information (NCBI). The CGView online server (http://stothard.afns.ualberta.ca/cgview_server/) was used to generate mitogenome maps. The resulting mitogenome sequences were deposited in GenBank (Table S1).

2.4. Mitogenome characterization and comparative analyses

The whole mitogenome alignment and individual alignments for PCGs, tRNAs and rRNAs were performed using the L-INSI algorithm in MAFFT v7.490 [73] within Geneious Prime (v.2024.0.2). The nucleotide base composition and the relative synonymous codon usage (RSCU) of each PCG was then calculated using MEGA v. 11.0 [74]. Strand asymmetry for all the studied mitogenomes was evaluated manually with the formula, AT-skew = [A − T] / [A + T] and GC-skew = [G − C] / [G + C]

[75]. To assess whether differences in nucleotide composition and skew metrics were statistically significant across calliphorid subfamilies, a Kruskal-Wallis test [76] was performed using species-level data. Phumosiinae was excluded due to insufficient sample size ($n = 1$). A post hoc Dunn's test [77] was used to identify significantly different subfamily pairs, with multiple testing correction applied using the Benjamini–Hochberg method [78]. All statistical tests were performed on R 4.4.1. All PCG alignments were tested for substitution saturation by plotting the number of transitions and transversions for each pairwise comparison against TN84 genetic distance using DAMBE 7 [79]. We also tested substitution saturation based on the Iss (simple index of substitution saturation) and Iss.c (critical Iss) values as an additional measure, as detailed in Xia et al. [80,81]. Iss and Iss.c define a threshold for significant data saturation and subsequently the reliability of phylogenetic inferences based on this data (Table S3). Non-synonymous nucleotide substitutions per non synonymous site (Ka), synonymous nucleotide substitutions per synonymous site (Ks), and evolutionary rate (Ka/Ks) of all 13 PCGs were also calculated for the newly generated sequences using DnaSP v6.12.03 [82]. The mitochondrial structure, including genome rearrangements, gene duplications and losses of all the studied species were assessed and compared manually in Geneious Prime v.2024.0.2. Repetitive elements and sequence motifs potentially associated with gene rearrangements were identified using REPuter [83].

2.5. Phylogenetic analyses

Each of the 13 PCGs were separately aligned using MAFFT under L-INSI algorithm [73], poorly aligned taxa and outliers were removed and then each PCG alignment was concatenated into a final 11,232 bp super matrix using SequenceMatrix [84]. The super matrix was then partitioned by gene and codon positions and analyzed using a Maximum likelihood (ML) framework in IQ-Tree (version 2.1.4-beta) [85]. ModelFinder [86] within IQ-Tree was used to determine the best partitioning scheme and evolutionary models based on the Akaike Information Criterion (AIC) (Table S4) [87]. Node support values for the majority-rule consensus ML tree were inferred with 10,000 replicates for both ultrafast bootstrapping [88] and SH-Like approximate likelihood ratio tests [89].

The final dataset was also analyzed using a Bayesian inference (BI) approach in ExaBayes v.1.5 [90], with eight simultaneous runs of four Markov Chain Monte Carlo (MCMC), each run for 1.5 million generations (sampled every 1000 generations, initial 25 % of samples discarded as burn-in). All priors were kept as default values. Effective sample size (ESS) and the potential scale reduction factor (PSRF) were assessed for statistical congruence using the in-built utility 'postProcParam', while topological congruence was evaluated via average standard deviation of split frequencies (asdsf). The extended majority-rule consensus tree was then constructed from each of the eight independent runs using the in-built utility 'consense'.

To investigate the influence of nucleotide composition on tree inference, we performed ML and BI analyses of two additional data sets using the methods outlined above: (1) An RY-coded phylogeny, which reduces compositional bias by recoding purines (A, G) as 'R' and pyrimidines (C, T) as 'Y,' and (2) A phylogeny with the 3rd codon position removed, a commonly used approach to mitigate substitution saturation [91–93].

For clarity, all the datasets are labeled as follows: (1) ML tree for the fully partitioned dataset with all codon positions (ML123), (2) ML tree for the partitioned dataset excluding the third codon position (ML12), (3) ML tree for the partitioned RY-coded dataset (MLRY), (4) BI tree for the fully partitioned dataset with all codon positions (BI123), (5) BI tree for the partitioned dataset excluding the third codon position (BI12), and (6) BI tree for the partitioned RY-coded dataset (BIRY).

To evaluate the support for the resolution of a monophyletic versus polyphyletic *Calliphora*, we performed a constrained ML analysis in IQ-Tree. We generated a constrained tree enforcing *Calliphora* monophyly and analyzed this under a ML framework with the same model parameters as the unconstrained tree. To assess whether the constrained tree was significantly worse than the best ML tree, we conducted topology tests comparing the alternate trees using the Approximately Unbiased (AU) [94], Shimodaira-Hasegawa (SH) [95], and Kishino-Hasegawa (KH) tests [96], implemented in IQ-TREE with 10,000 RELL bootstrap replicates (-au option) [97].

The tree graphics for all phylogenetic analyses were visualized using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) and edited using Inkscape 1.3.2 (available at https://inkscape.org).

3. Results and discussion

3.1. Mitogenome structure, organization and composition

Our study substantially expands the available mitogenomic dataset for the family Calliphoridae by generating 96 mitochondrial genome assemblies, including 73 complete/near-complete and 23 partial mitogenomes (missing either rRNA or tRNA genes, but retaining all 13 protein-coding genes; Table S2). All mitogenomes displayed a conserved, circular, double-stranded structure, with typical gene content and order for Diptera: 13 protein-coding genes (PCGs), 22 tRNAs, 2 rRNAs, and a non-coding AT-rich control region (Fig. 1). Genome lengths ranged between 14 kb and 19 kb, consistent with known dipteran mitogenomes [6,7,36,98].

3.1.1. Base composition and compositional biases

All mitogenomes were strongly A + T-biased (75.3 %–82.8 %), a pattern well documented in Diptera, including calliphorids [5,7,36,99] (Table S2). However, significant heterogeneity in nucleotide composition was detected across subfamilies. Kruskal–Wallis tests revealed statistically significant inter-subfamily differences for both A + T % ($\chi^2 = 18.83$, $p = 0.004$) and G + C % ($\chi^2 = 18.73$, $p = 0.005$) (Table S5). Rhinophorinae exhibited the highest A + T content (mean = 80.4 %, SD = 2.352), while Luciliinae showed the lowest (mean = 76.9 %, SD = 0.7) (Tables 2, S6). These trends were also evident in PCGs, where A + T content ranged from 75 % (SD = 0.772) in Luciliinae to 78.2 % (SD = 0.593) in Rhinophorinae (Table 2), with significant inter-subfamily variation (for both, A + T % and G + C %, $\chi^2 = 17.83$, $p = 0.007$) (Table S5). Post hoc Dunn's tests identified marginal significant pairwise differences between Luciliinae–Rhinophorinae for both complete mitogenomes ($p = 0.025$) and PCGs ($p = 0.005$) (Tables S5, S6). These base composition differences likely reflect lineage-specific mutational or selective pressures, potentially linked to ecological or metabolic divergence across subfamilies [10].

Compositional biases (AT-skew and GC-skew) play an essential role in mitogenomic stability and evolution [9,75,100]. Across Calliphoridae, we observed a general pattern of positive AT skew and negative GC skew in the complete mitogenomes, which is inverted in the PCG regions (Table 2), consistent with prior studies demonstrating strand-specific mutational asymmetries during replication [7,23,99].

Kruskal–Wallis tests revealed significant inter-subfamily differences for AT-skew (complete mitogenome: $\chi^2 = 17.02$, $p = 0.0092$; PCGs: $\chi^2 = 13.33$, $p = 0.038$) and GC-skew ($\chi^2 = 18.87$, $p = 0.0044$), though GC-skews for PCGs were not significant ($\chi^2 = 11.46$, $p = 0.075$) (Table S5). Notably, Rhinophorinae significantly differed from Bengaliinae and Luciliinae in both AT- and GC-skew metrics for whole mitogenomes (AT-skew: $p = 0.008$, $p = 0.05$, respectively; GC-skew: $p = 0.039$, $p = 0.02$, respectively), while differences in PCGs were less pronounced (Table S6).

These strand asymmetry metrics, though sometimes marginal in pairwise comparisons, point to biologically meaningful variation. They may reflect differential replication dynamics, mutation pressures, or even constraints imposed by gene order or the position of the origin of replication [100]. Prior work has linked compositional skews to strand-biased mutation rates and genome rearrangement events in other metazoans [9,10], highlighting the role of compositional biases in

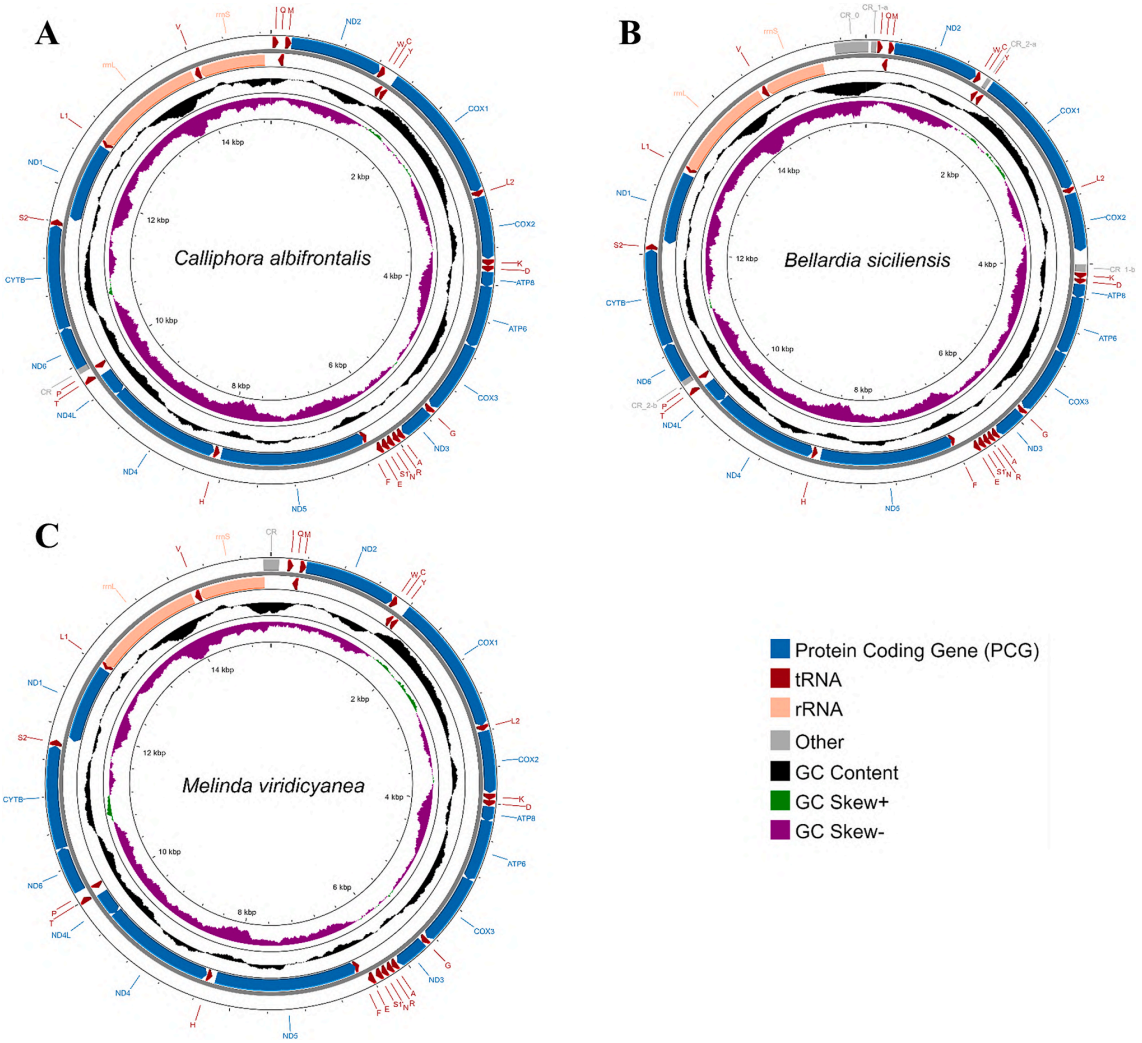

Fig. 1. Representative Calliphorinae mitogenomes of *Calliphora albifrontalis* Malloch (A), *Bellardia siciliensis* Villeneuve (B) and *Melinda viridicyanea* Robineau-Desvoidy (C). The direction of gene transcription is indicated by arrows within the strands. Genes in the outer circle are encoded by the major coding strand (5′–3′), and inner circle genes are encoded by the minor strand (3′–5′). Transfer RNA are represented by the single letter IUPAC-IUB abbreviations for their corresponding amino acid. Names and colors follow the legend available in the lower right.

mitogenomic evolution beyond phylogenetics, although no such direct correlation was found within Calliphoridae in our dataset (refer to Section 3.4).

Although compositional biases did not appear to strongly influence the phylogenetic reconstruction in this study (refer to Section 3.5), they may still provide insight into genome stability and may reflect underlying evolutionary processes shaping mitogenome architecture. For instance, lineage-specific differences in base composition and skews may be tied to metabolic demands, replication mechanisms, or structural genome constraints, particularly in taxa with distinct ecological niches or life history strategies [9–11].

While these patterns offer valuable evolutionary insights, given the limited sample size for certain subfamilies (e.g., Rhiniinae, Rhinophorinae, $n = 3$), future work incorporating more complete mitogenomes from underrepresented lineages will be essential to validate and further explore these trends to strengthen comparative analyses. Complete details on mitogenome length, accession numbers, base composition, and missing genes are summarized in Table S2.

3.2. Codon usage

Across the Calliphoridae, most protein-coding genes (PCGs) began with canonical ATN start codon, except for COI, ND1 and ND5, which utilized non-standard start codons (TCG/CGA/CAA, TTG, GTG, respectively), reflecting common deviations reported in other Dipteran studies [3,24,25,36,99]. Similarly, while most PCGs terminated with complete stop codons (TAA/TAG), truncated stop codons (TA/ T) were detected in COI, COII, ND4, ND5 and CYTB in a subset of species. These incomplete stop codons are presumed to be restored via post-transcriptional polyadenylation, a widespread mechanism in insect mitochondrial gene expression [5,7,25,98,99].

Relative Synonymous Codon Usage (RSCU) is widely used to measure codon usage bias, or the preference to use one codon over another to encode a particular amino acid. Our analysis detected unequal usage

Table 2
Nucleotide composition of Calliphoridae mitogenomes. For each subfamily, mean ± standard deviation is reported for all nucleotide composition and skew parameters to reflect intra-subfamily variation, except for Phumosiinae, which is represented by a single specimen and thus lacks a standard deviation.

Gene	Feature	Ameniinae	Bengaliinae	Calliphorinae	Chrysomyinae	Luciliinae	Phumosiinae	Rhiniinae	Rhinophorinae	Calliphoridae
Entire mitochondrial genome	A + T %	78.5 ± 0.4	77.325 ± 0.236	77.193 ± 1.09	77.066 ± 0.153	76.9 ± 0.7	77.4	78.766 ± 0.351	80.4 ± 2.352	77.4 ± 1.226
	G + C %	21.466 ± 0.503	22.725 ± 0.189	22.802 ± 1.084	22.866 ± 0.115	23.066 ± 0.751	22.6	21.233 ± 0.351	19.566 ± 2.303	22.5 ± 1.223
	AT-skew	0.01 ± 0.04	0.03 ± 0.008	0.0013 ± 0.022	0.009 ± 0.022	0.02 ± 0.003	0.036	0.015 ± 0.036	−0.034 ± 0.016	0.002 ± 0.024
	GC-skew	−0.059 ± 0.233	−0.19 ± 0.016	−0.017 ± 0.169	−0.06 ± 0.197	−0.19 ± 0.006	−0.194	−0.064 ± 0.195	0.173 ± 0.015	−0.031 ± 0.171
Protein-coding genes	A + T %	76.5 ± 0.194	75.9 ± 0.487	75.8 ± 0.843	75.8 ± 0.387	75 ± 0.772	75.4	77.0 ± 0.418	78.2 ± 0.593	75.9 ± 0.92
	G + C %	23.5 ± 0.194	24.1 ± 0.487	24.2 ± 0.843	24.2 ± 0.387	24.9 ± 0.772	24.6	22.9 ± 0.418	21.8 ± 0.593	24.1 ± 0.92
	AT-skew	−0.15 ± 0.006	−0.154 ± 0.006	−0.15 ± 0.004	−0.155 ± 0.007	−0.154 ± 0.004	−0.145	−0.148 ± 0.004	−0.14 ± 0.006	−0.151 ± 0.005
	GC-skew	0.004 ± 0.015	0.021 ± 0.005	0.024 ± 0.012	0.024 ± 0.005	0.012 ± 0.016	0.016	0.039 ± 0.012	0.018 ± 0.006	0.021 ± 0.012

of 62 codons across all Calliphoridae mitogenomes, including Calliphorinae (Figs. 2, S2). Our analysis revealed a clear preference for codons ending in A or U (RSCU >1), particularly in synonymous codons encoding leucine (UUA), serine (UCU), and glycine (GGA). This A/U-ending bias corresponds with the overall high A + T content of calliphorid mitogenomes and mirrors patterns observed in other insect taxa [6,7,11,99].

This codon usage pattern is likely driven by both neutral mutational biases and functional selection. The A + T-rich context of mitochondrial genomes predisposes them to favor A/U-ending codons, which may reduce the metabolic cost of replication and transcription [10,101,102]. However, to determine whether selection also plays a role in shaping codon usage, we examined the availability of corresponding tRNA anticodons (Table S7). Frequently used codons (e.g., UUA-Leu, UCU-Ser, GGA-Gly) were generally complementary to the most abundant mitochondrial tRNA anticodons, suggesting selection for translational efficiency via optimal codon–anticodon pairing. Thus, codon usage in Calliphoridae mitogenomes appears to reflect a dual influence of mutational pressures (e.g., AT bias) and selective forces (e.g., energy-efficient translation and tRNA compatibility), consistent with broader evolutionary trends observed in insect mitochondrial evolution [103,104].

3.3. Protein coding gene evolutionary rate

We examined substitution rates across 13 mitochondrial PCGs to assess evolutionary rate variation among Calliphoridae subfamilies. Overall, synonymous substitution rate (Ks) was largely consistent across subfamilies, with a slight acceleration in Ameniinae (Ks = 0.50) and the lowest rate in Calliphorinae (Ks = 0.32) (Fig. 3B). In contrast, non-synonymous substitution rates (Ka) showed greater variation, ranging from 0.09 (Rhinophorinae) to 0.014 (Luciliinae). In particular, Rhinophorinae exhibed 2.1× faster than the average Ka value, suggesting relaxed purifying selection or adaptative evolution in this lineage (Fig. 3A). As Phumosiinae was only represented by a single specimen, substitution rates in this subfamily could not be analyzed.

The Ka/Ks ratio (ω), commonly used to evaluate selective pressure on protein-coding genes, further supported lineage-specific rate heterogeneity [105]. Subfamilies such as Ameniinae (ω = 0.32) and Rhinophorinae (ω = 0.25) exhibited elevated ω values compared to other subfamilies (average ω = 0.13), indicating relatively faster evolutionary rates and suggesting potential adaptation to distinct ecological niches—snail parasitism in Ameniinae and woodlouse parasitoidism in Rhinophorinae (Fig. 3C). In contrast, Luciliinae (ω = 0.03), Chrysomyinae (ω = 0.04) and Calliphorinae (ω = 0.06) displayed lower than

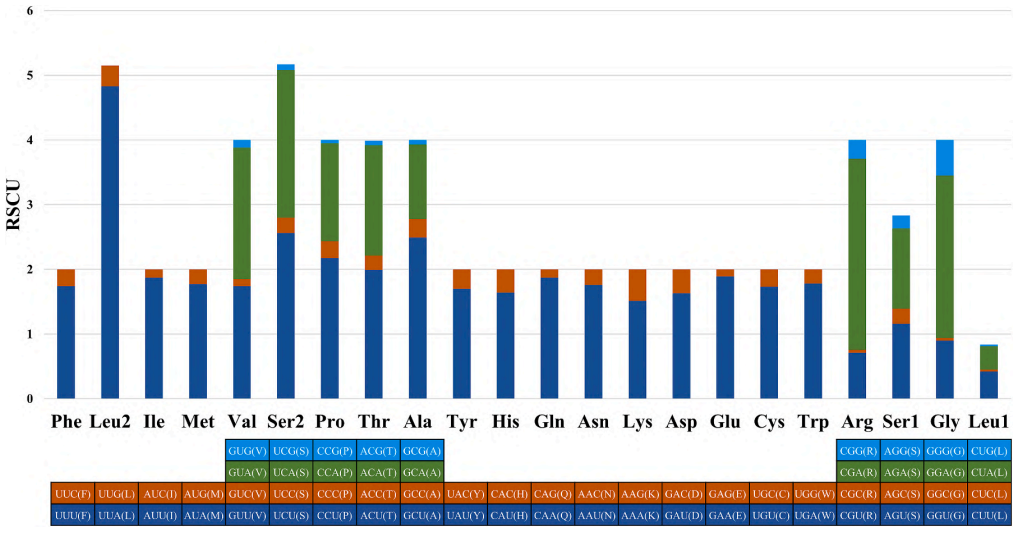

Fig. 2. Relative synonymous codon usage (RSCU) of the mitochondrial protein coding genes of Calliphoridae.

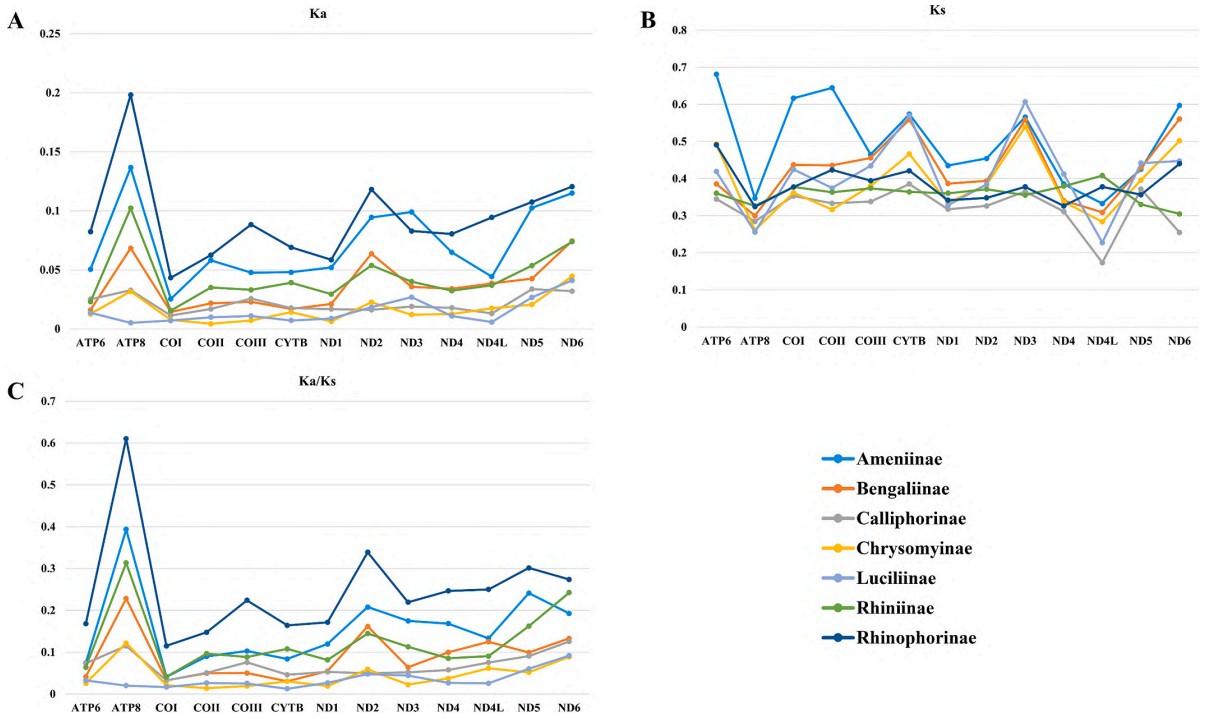

Fig. 3. Synonymous (A) and non-synonymous (B) substitution rates and the Ka/Ks ratios (C) of protein coding genes in blowflies. Abbreviations: ATP6, ATP8, ATP synthetase subunits 6 and 8 genes; CYTB, cytochrome oxidase b gene; COI–COIII, cytochrome oxidase c subunit 1–3 genes; Ka, non-synonymous substitutional rate; Ks, synonymous substitutional rate; ND1–6, ND4L, NADH dehydrogenase subunits 1–6 and 4L genes.

average ω values, implying comparatively stronger purifying selection and functional conservation of the mitochondrial genes within these lineages (Fig. 3C).

Gene-specific substitution rates revealed that ATP8 (ω = 0.25), ND6 (ω = 0.16), ND2 (ω = 0.144) and ND5 (ω = 0.143) exhibited elevated evolutionary rates compared to the genome-wide average (ω = 0.10), with ATP8 and ND6 evolving significantly faster than other mitochondrial genes (2.5× and 1.6× respectively), suggesting these genes may be evolving under weaker purifying or relaxed selection [106]. Conversely, COI exhibited the lowest ω (0.04) supporting its continued use as a conserved phylogenetic marker under strong functional constraints [105,106].

Interestingly, the elevated Ka and ω values in Rhinophorinae correlated with high A + T content, hinting at a possible link between nucleotide composition bias and substitution dynamics. [107]. This may reflect lineage-specific metabolic demands or adaptive pressures associated with the specialized parasitoid lifestyle of species in this subfamily, such as host-specific interactions or energy-intensive reproduction strategies.

Although composition biases (e.g., AT/GC skew, RSCU, Ka/Ks) were not explicitly included in our phylogenetic reconstruction, we evaluated their potential impact using alternative data treatments, such as RY coding and exclusion of third codon positions (refer to Section 3.5). These approaches revealed no significant effect on overall tree topology, suggesting the robustness of our phylogenetic framework. Nonetheless, substitution rates and codon usage patterns offer important evolutionary insights and highlight the need for integrated genomic approaches to better understand the functional and ecological diversity within Calliphoridae.

3.4. Gene rearrangements

3.4.1. Mitochondrial rRNA and protein coding gene rearrangement

While most calliphorid mitogenomes followed the conserved ancestral pancrustacean insect gene arrangement [2] (Fig. 1), we identified several instances of independent gene rearrangements within the family. These events included major translocations, strand switches, gene duplications, gene losses and fragmentations. Importantly all gene rearrangements were restricted to the subfamily Calliphorinae, highlighting the structural variability within this subfamily relative to other blowfly lineages.

Notably, *Calliphora varifrons* Malloch (PQ594010) exhibited major rearrangements involving inverse transposition (both, gene moved to another location and coding direction/strand switched) of -rrnL, rrnS, trnV (modified order) instead of -rrnL, -trnV, -rrnS (most common order) (Fig. 4). Furthermore, the rrnL gene was split into two non-contiguous fragments on the negative strand: a smaller upstream fragment (1017–923 bp) and a larger downstream fragment (15498–16,250 bp). Comparison of synteny with complete mitogenomes from other closely related *Calliphora* species did not reveal similar rearrangements, suggesting that the gene structure in *C. varifrons* is likely a rare, lineage-specific event. We propose Tandem Duplication–Random Loss (TDRL) as the mechanism for this rearrangement, which is supported by our REPuter analysis [83] which identified a 106 bp AT-rich direct repeat flanking the two rrnL fragments, as well as palindromic and complementary repeats near rrnS and trnV [108]. While major rearrangements have been reported in other Hymenopteran mitogenomes [17,109], this finding is the first report of major mitochondrial rearrangement in Calliphoridae, and only the second in Cyclorrhapha (*Admontia podomyia* Brauer & Bergenstamm, Tachinidae [5]).

Another unusual structural feature was identified in *Calliphora uralensis* Villeneuve (PQ594004), where the ATP6 gene was fragmented

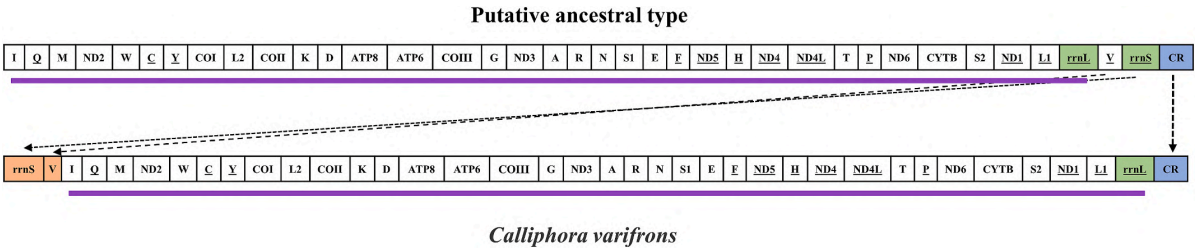

Fig. 4. The comparison of the mtDNA gene rearrangement between *Calliphora varifrons* Malloch and putative ancestral type (*Drosophila yakuba* Burla). Underlined genes are encoded on the minor strand. Gene translocations are shown in orange, two rRNA subunits (lrRNA and srRNA) are in green; control regions (CR) are in blue. The purple underline represents similar gene clusters between *Calliphora varifrons* and the putative ancestral type.

into two parts, with the second fragment relocated to a region adjacent to the origin of replication. In addition, a strand switch in the CYTB gene was detected in *Polleniopsis mongolica* Séguy (GenBank accession: MN131052), though this rearrangement was not shared by other conspecifics.

Although earlier studies have proposed mitochondrial gene rearrangements as potential synapomorphies useful for resolving taxonomic groups [2], our findings indicated that such events in Calliphoridae are largely independent, rather than shared derived traits. These rearrangements likely stem from repeat-driven recombination and local structural instability. Therefore, although gene rearrangements remain informative for mitochondrial genome evolution, they should be interpreted with caution when used for phylogenetic inference within Calliphoridae.

3.4.2. tRNA gene rearrangement

Previous studies have shown that mitochondrial tRNAs exhibit greater structural and positional variability than rRNAs or PCGs, accounting for up to 80 % of all mitochondrial rearrangements [110,111]. Our findings support this observation, with tRNA genes involved in ~75 % of the rearrangements detected in Calliphoridae. Although earlier investigations in blowflies reported tRNA duplications, these were often limited to the control region and focused on a small number of genera [21–24]. In contrast, our study–based on broad sampling across Calliphoridae revealed tRNA duplications/loss in several species, including *Bellardia bayeri* Jacentkowský, *Bellardia pandia* Walker, *Calliphora subalpina* Ringdahl, *Calliphora latifrons* Hough, *Calliphora calliphoroides* Rohdendorf, *Onesia zumpti* Schumann and *Onesia accepta* Malloch (Table 3). Notably, in *B. bayeri* (OX493271) we identified three copies of trnI: two identical and one divergent. The divergent copy was translocated between trnE and trnF, forming a novel gene cluster (trnE-trnI-trnF), which represents the first such rearrangement reported in Calliphoridae. This expanded trnI copy number may reflect increased demand for metabolic efficiency, as reported in other insects [112]. Interestingly, many of the duplicated tRNAs (e.g., trnI) correspond to frequently used codons (e.g., AUU for Ile), and their anticodons rely on wobble base pairing for recognition. This suggests that selection for enhanced translational efficiency may play a role in retaining functionally duplicated tRNAs (Fig. 2; Table S7) [113].

Table 3
The tRNA duplication/loss in mitochondrial genomes of Calliphorinae.

Genera	Species	Accession No.	Duplication (D)/Loss(L)	Duplication (D)/ Loss(L) in conspecifics
Bellardia	*Bellardia bayeri*	PQ635411	L:trnF, D: Partial trnI	D: trnI (3 copies), trnQ, partial trnM (OX493271)
	Bellardia pandia	PQ664929	L:trnI	D:trnQ, trnM (OU696535)
Calliphora	*Calliphora calliphoroides*	PQ594007	NA	D:trnI (MK893471)
	Calliphora latifrons	PQ593991	D:trnI	NA
	Calliphora subalpina	PQ593982	D:trnI	NA
Onesia	*Onesia accepta*	PQ635405	D:trnF	NA
	Onesia zumpti	PQ594003	D:trnF	NA

Sequence divergence among duplicated tRNAs in *Bellardia* Robineau-Desvoidy, *Calliphora* and *Onesia* Robineau-Desvoidy indicates a non-homologous origin of these duplications, in contrast to the conserved, synapomorphic duplications seen in *Chrysomya* [25]. While many duplicated or partially duplicated tRNAs may be non-functional or become pseudogenes, others may retain functional roles and contribute to protein synthesis [114]. In our dataset, however, *Chrysomya* species showed no such tRNA duplication synapomorphies, contradicting earlier reports [25]. Similarly, *Calliphora dubia* Macquart (PQ593997) exhibited no trnI duplication, aligning with the ancestral gene order, deviating from earlier findings [22].

Our results suggest a possible role of habitat-driven selective pressure in shaping mitochondrial genome evolution based on variation observed between geographically distinct populations of the same species, as previously reported [16]. For instance, *B. pandia* from the United Kingdom (OU696535) exhibited multiple tRNA duplications, while a Polish specimen (PQ664929) showed none. Similarly, *C. subalpina* from Russia (PQ593982) carried duplicated trnI, but this rearrangement was absent in specimens from Poland (PQ593981) and Germany (PQ594009). Although the frequency of such random rearrangement events is largely unknown, our findings add to the evidence that multiple independent rearrangements can occur even within a single species [22,115]. Overall, the inconsistent tRNA rearrangements within and across species, especially among geographically distinct populations, strongly suggest that these are homoplasies, likely arising independently through neutral evolutionary processes, rather than shared ancestry (refer to Section 3.4.1). Interestingly, our analyses found no significant association between gene rearrangement and compositional biases, strand asymmetry, or selective pressure, implying a largely neutral origin. This finding contrasts with prior studies suggesting that mitochondrial rearrangements in insects may reflect adaptive responses to selection [9,16].

3.5. Phylogenetic inference

Our phylogenetic dataset comprised all 13 mitochondrial PCGs from 107 mitogenomes, representing 84 species across all eight calliphorid subfamilies (Table S1). Analysis of this dataset produced fully resolved and mostly concordant ML and BI phylogenies, regardless of data, partitioning schemes or coding strategies (Figs. 5, S3–S7). All analyses strongly supported the monophyly of Calliphoridae (Figs. 5, S3–S7) and shared a similar topology to Yan et al. [30], including the treatment of Rhiniinae and Rhinophorinae as valid calliphorid subfamilies. Our analyses also strongly supported the family rank of Mesembrinellidae and its sister relationship to Ulurumyiidae, consistent with previous

molecular studies [30,34,36,39,41].

3.5.1. Subfamily relationships in Calliphoridae

Our study includes representatives of all recognized calliphorid subfamilies, thereby providing a framework for assessing subfamilial relationships. All calliphorid subfamilies were recovered as monophyletic, consistent with previous studies, except for Phumosiinae, which was represented by a single species in our analyses [25,30,34,36,116]. The overall subfamily structure recovered in our partitioned dataset with all codon positions (both ML and BI) largely aligned with Yan et al. [30], except for the sister relationship between Chrysomyinae and Phumosiinae, which was not recovered in four of our datasets (ML123, BI123, ML12, BI12). Notably, our RY-coded analyses (both ML and BI) recovered Chrysomyinae and Phumosiinae as sister taxa, aligning with the primary hypothesis of Yan et al. [30]. *Phumosia promittens* Walker was recovered as sister to all other Calliphoridae (ML123, BI123, BI12), contrasting with previous morphological [47,117] and molecular evidence [44]. This suggests that compositional biases may influence the placement of Phumosiinae, highlighting the impact of different data treatments on phylogenetic outcomes.

Calliphorinae was recovered as monophyletic across all data treatments, forming a well-supported sister clade with Luciliinae, a relationship widely supported in previous molecular [25,34,36,38,99,116] and morphology-based analyses [45,118]. We consistently recovered the former Melanomyinae nested inside the Calliphorinae, with strong nodal support, reinforcing prior evidence from morphological [32,45,119], mito-nuclear [34,41], protein-encoding ultraconserved elements (UCEs) [44] and transcriptomic datasets [30]. The Neotropical representative of the former Toxotarsinae was consistently recovered as sister to all other Calliphorinae, a pattern congruent with recent molecular studies [30,44] but contrasting morphological analyses that placed it as sister to Chrysomyinae [40,45]. This basal placement of the South American endemic Toxotarsinae could reflect an early divergence within the group and potentially supports a Southern Hemisphere origin of Calliphorinae. Chrysomyinae was recovered as monophyletic with strong support in all datasets, forming a sister group to Calliphorinae+Luciliinae, which is in agreement with previous findings [34,36,43,51,99].

Our study supported the subfamily status of Rhiniinae, which consistently formed a well-supported clade with the monophyletic Bengaliinae (except in ML12), aligning with previous studies [30,41,44,116]. Similarly, Rhinophorinae was also confirmed as a valid calliphorid subfamily, forming a strongly supported sister group with Ameniinae in all datasets (except in ML12) [30,37,116]. Interestingly, the placement of *Eurychaeta palpalis* Robineau-Desvoidy (former Helicoboscinae) varied across analyses: grouping with Rhinophorinae (ML123, ML12, BI12), Ameniinae (BI123), or forming an independent clade with low nodal support (MLRY, BIRY). This phylogenetic instability mirrors previous studies—Kutty et al. [34], which placed *E. palpalis* near Rhinophorinae using mito-nuclear dataset but lacked Ameniinae representatives, while Yan et al. [30] recovered it as sister to Ameniinae using transcriptomic data, leading to the taxon's reassignment. The shifting placement of *E. palpalis* may be due to long-branch attraction, rapid mitochondrial evolution, or compositional bias, which can be exacerbated by sparse or uneven taxon sampling. Additionally, incomplete lineage sorting (ILS) [120] could contribute to conflicting phylogenetic signals, especially if these subfamilies underwent rapid divergence, a phenomenon well documented in blowflies [25,121–123]. The consistently low nodal support and topological instability surrounding *E. palpalis* across all datasets highlights the need for denser taxon sampling and multi-locus phylogenomic analyses, particularly those incorporating nuclear markers, to robustly assess its placement and broader subfamilial relationships.

3.5.2. Phylogenetic relationships within Calliphorinae

Within Calliphorinae, both ML and BI phylogenies were largely congruent across all data treatments, with the exception of a few clades showing inconsistent or weak nodal support (discussed below). The genus *Calliphora*, which had the highest species representation in our dataset, was recovered as paraphyletic, consistently clustered into two major clades across all analyses. These clades were seperated by a grouping of Melanomyinae+*Pericallimyia* Villeneuve, supporting the recent synonymization of Melanomyinae with Calliphorinae [30,44]. The first major *Calliphora* clade, consisting mostly of Northern Hemisphere species, was rendered paraphyletic by the inclusion of species from the genera *Cynomya*, *Cyanus* and *Cynomyiomima*. The second clade consistently emerged with Australasian species of *Calliphora*, including species of the predominantly Palearctic, multilarviparous genera *Bellardia*, *Onesia* and *Polleniopsis* Townsend. The paraphyly of *Calliphora* was further confirmed using a constrained ML phylogenetic analysis. Comparison with the unconstrained ML tree using AU, SH, and KH tests (Table S8) showed a significantly lower log-likelihood for the monophyly-constrained tree (ΔlogL = 168.69). All topology tests strongly rejected *Calliphora* monophyly (p-AU = 1.81e-37, p-KH = 0.0000, p-SH = 0.0000), supporting previous molecular evidences [36,46,52].

Both, *Cyanus elongata* Hough and *Cynomyiomima stackelbergi* Rohdendorf had unstable placements, with very low nodal support across all ML and BI trees (Figs. 5, S3–S7). In contrast, *Cynomya* species consistently emerged, with strong support, within the first splits of the Northern Hemisphere *Calliphora* clade, as sister to *Calliphora latifrons* across all analyses (except in ML12 and BI12) (Figs. 5, S3–S7). This result aligns with previous mitochondrial and nuclear Sanger analyses that support a close relationship between *Cynomya mortuorum* Linnaeus and *Calliphora vicina* Robineau-Desvoidy [46,124]. However, adult morphology has traditionally maintained *Cynomya* as distinct, based on apomorphic characters: absence of a projecting cornu on the hypophallus, unique head coloration, thoracic chaetotaxy (e.g., acrostichal setae count), and genitalic and abdominal microtomentum patterns [32,125]. However, adult wing morphometrics reveal overlapping traits between these taxa [126], further confirming the placement of *Cynomya* within *Calliphora*, as mentioned in preimaginal taxonomic keys [127]. Similarly, larval morphology, particularly in the the first instar, reveals significant similarities between *C. vicina* and *Cy. mortuorum*, with nearly indistinguishable cephaloskeletal shape and spinulation patterns [128]. Our mitogenomic phylogeny reinforces this molecular affinity, placing *Cynomya* firmly within the *Calliphora* clade (Fig. 5), and corroborates earlier genetic findings [46,124]. However, the observed morphological-molecular discordance, particularly between adult and larval traits, highlights a potentially complex evolutionary history, possibly shaped by retained ancestral polymorphism or morphological convergence. Taken together, current mitogenomic data, supported by previous nuclear and larval morphological analyses, provide substantial evidence for the synonymization of *Cynomya* within *Calliphora*. However, we refrain from proposing formal taxonomic revisions until further integrative studies, incorporating detailed comparative morphology across life stages, nuclear genomic data, and expanded taxon sampling (especially for problematic genera such as *Cyanus* and *Cynomyiomima*) can be completed.

Alternative partitioning strategies such as RY coding and third codon exclusion have been widely used in insect phylogenetics to mitigate compositional bias and rate heterogeneity [91–93]. However, these approaches have shown mixed effects depending on the dataset and taxonomic group [91,129,130]. In our study, both approaches resulted in one or more of the following: reduced tree stability, weaker nodal support, or biologically implausible placements for certain taxa (Figs. S4–S7). These results suggest that while compositional biases exist, excluding third codon positions or applying RY coding does not necessarily improve mitogenome-based phylogenetic inference in the Calliphoridae. This is consistent with previous studies showing that including the third codon position in PCGs does not negatively impact phylogenetic reconstruction in insects [131], while excluding it often

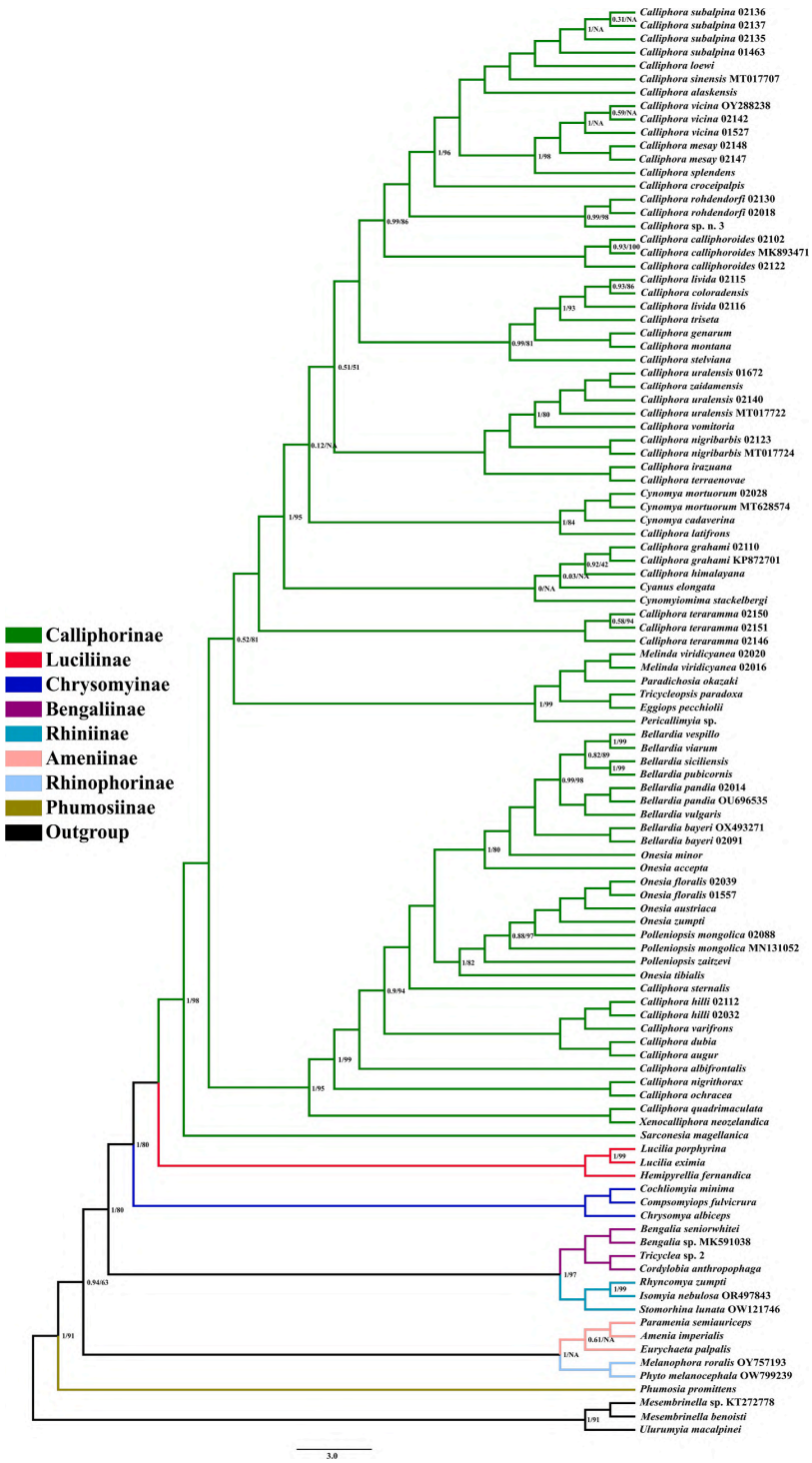

Fig. 5. Mitochondrial genome phylogeny of Calliphoridae, using Mesembrinellidae and Ulurumyiidae as outgroups. The phylogenetic tree is based on nucleotide datasets for 13 PCGs (including all codon positions) from the mitogenomes of 84 species (107 taxa) using Maximum Likelihood estimation in IQ-tree (ML123) under AIC-based model selection and Bayesian inference using ExaBayes (BI123). Nodal support is provided for nodes with support values below 1.0 for ExaBayes posterior probabilities or below 100 % for IQ-TREE ultrafast bootstrap support, respectively. 'NA' in the position of a support value indicates clades not resolved in the respective analysis. The voucher codes or Genbank accession numbers are included along with the species with more than one specimen, for clarity. Colors of the clades represent the corresponding calliphorid subfamilies as indicated in figure legends.

leads to loss of phylogenetic signal or introduces unexpected topologies (i.e., in Diptera, including Calliphoridae [36,130]). Additionally, the nucleotide substitution saturation analysis further confirmed that third codon positions were not fully saturated, supporting their retention in our primary dataset.

Given these findings, we recommend the partitioned analysis including all three codon positions as the optimal dataset, as it provided the most stable and well-supported phylogeny and best aligned with morphological expectations and prior molecular evidence in Calliphoridae. Although our analyses account for compositional biases through partitioned models and alternate data treatments, compositional heterogeneity remains a recognized challenge in insect mitochondrial phylogenetics, particularly for the resolution of deep nodes [129,132–134]. Future studies integrating nuclear genome data, site-heterogeneous models and denser taxon sampling will be critical for addressing these challenges.

4. Summary

In this study, we generated the most comprehensively sampled mitogenomic dataset to date for the Calliphoridae, including first ever mitogenomes for 68 species. This study is the first to combine comparative mitogenomics and phylogenomics across all recognized calliphorid subfamilies, utilizing the largest taxon sampling ever assembled for the group. Despite this expanded sampling, some Calliphorinae genera remain underrepresented, largely due to the limited availability of high-quality genomic data and/or specimens, as many species are rare, regionally restricted and can be difficult to collect. Future studies incorporating targeted field sampling and leveraging advanced sequencing approaches, such as ultra-conserved elements (UCEs) or hybrid-capture techniques, may help fill these gaps and improve taxonomic representation across the family. Our study offers new insights into mitochondrial genome architecture in Calliphoridae, particularly the patterns of gene rearrangements, which were found exclusively within Calliphorinae. Although no consistent pattern of rearrangement emerged among Calliphorinae genera or species, their diverse but infrequent occurrence in closely related taxa could be informative for understanding the evolutionary and speciation dynamics within the subfamily. Notably, the gene translocations identified in *C. varifrons* highlight the need for further investigations into mitochondrial genome structure, ideally using multiple specimens across its geographic range. We also found that Calliphorinae exhibits relatively slow mitochondrial evolutionary rates compared to most other calliphorid subfamilies, suggesting stronger purifying selection on its mitogenomes. Phylogenetic inference based on our mitogenomic data supports a monophyletic Calliphoridae and aligns with the eight-subfamily system proposed by Yan et al. [30]. Looking forward, although mitogenomes proved highly informative in this study, we recommend that future phylogenetic work integrate nuclear genomic data to address limitations such as compositional bias and low genetic variability, especially in higher-level phylogenetic analysis. This is particularly important given the potential for mitochondrial introgression and limited phylogenetic resolution of mitochondrial markers alone, as reported in previous studies [25,34,46].

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CRediT authorship contribution statement

Drashti R. Parmar: Writing – review & editing, Writing – original draft, Validation, Investigation, Data curation, Conceptualization, Formal analysis, Methodology, Visualization. **Nikolas P. Johnston:** Writing – review & editing, Writing – original draft, Validation, Investigation, Data curation, Methodology, Supervision. **Krzysztof Szpila:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization, Project administration, Resources, Data curation.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT in order to improve readability. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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Table S1. Collection information of specimens used for DNA extraction in the present study. A "•" and "*" represents the specimens sequenced in this study and species with partial mitochondrial genome, respectively.

ID	Family	Subfamily	Genera	Species	Location	Latitude	Longitude	GenBank Accession	Source
KEIB_DIP_02070	Calliphoridae	Calliphorinae	*Calliphora*	sp. n. 3	Fajã da Ovelha, Madeira, Portugal	32.80	-17.16	PQ593971	•
KEIB_DIP_02099	Calliphoridae	Calliphorinae	*Calliphora*	*alaskensis*	West Vancouver, BC, Canada	49.37	-123.24	PQ593972	•
KEIB_DIP_02101	Calliphoridae	Calliphorinae	*Calliphora*	*augur* *	Canberra Central, Black Mountain, Australia	-35.27	149.09	Appendix 1	•
KEIB_DIP_02102	Calliphoridae	Calliphorinae	*Calliphora*	*calliphoroides*	Kabansky District, Buryatia, Russia	51.38	105.17	PQ593973	•
KEIB_DIP_02104	Calliphoridae	Calliphorinae	*Calliphora*	*coloradensis*	Page Springs, Harney County, OR, USA	42.80	-118.86	PQ593974	•
KEIB_DIP_02109	Calliphoridae	Calliphorinae	*Calliphora*	*genarum*	Kabansky District, Buryatia, Russia	51.40	105.08	PQ593975	•
KEIB_DIP_02110	Calliphoridae	Calliphorinae	*Calliphora*	*grahami*	Kangwon, North Korea	38.68	128.18	PQ593976	•
KEIB_DIP_02112	Calliphoridae	Calliphorinae	*Calliphora*	*hilli*	Wollemi NSW 2330, Australia	-32.96	150.43	PQ593977	•
KEIB_DIP_02113	Calliphoridae	Calliphorinae	*Calliphora*	*himalayana*	Botapathri Woods, Forest Block, Gulmarg, India	34.08	74.34	PQ593978	•
KEIB_DIP_02115	Calliphoridae	Calliphorinae	*Calliphora*	*livida* *	Milwaukee Bay, Wisconsin, USA	43.03	-87.87	Appendix 1	•
KEIB_DIP_02116	Calliphoridae	Calliphorinae	*Calliphora*	*livida* *	Baker School District 5J, OR, USA	44.81	-117.89	Appendix 1	•
KEIB_DIP_02121	Calliphoridae	Calliphorinae	*Calliphora*	*montana* *	Tomponskiy Ulus, Sakha Republic, Russia	63.8	137.88	Appendix 1	•
KEIB_DIP_02122	Calliphoridae	Calliphorinae	*Calliphora*	*calliphoroides*	Nakajima, Sakura Ward, Saitama, Japan	35.86	139.62	PQ594007	•
KEIB_DIP_02123	Calliphoridae	Calliphorinae	*Calliphora*	*nigribarbis* *	Himonya, Meguro City, Tokyo, Japan	35.61	139.68	Appendix 1	•

KEIB_DIP_02124	Calliphoridae	Calliphorinae	*Calliphora*	*nigrithorax*	Seven Mile Beach, Berry NSW 2535, Australia	-34.79	150.77	PQ593979	•
KEIB_DIP_02130	Calliphoridae	Calliphorinae	*Calliphora*	*rohdendorfi*	Gorod Gelendzhik, Krasnodar Krai, Russia	44.53	38.56	PQ593980	•
KEIB_DIP_02131	Calliphoridae	Calliphorinae	*Calliphora*	*splendens* *	Guía de Isora, Santa Cruz de Tenerife, Spain	28.26	-16.74	Appendix 1	•
KEIB_DIP_02132	Calliphoridae	Calliphorinae	*Calliphora*	*stelviana* *	Vargvägen 3, 830 19 Storlien, Sweden	63.31	12.10	Appendix 1	•
KEIB_DIP_02135	Calliphoridae	Calliphorinae	*Calliphora*	*subalpina*	Gmina Czorsztyn, Poland	49.39	20.39	PQ593981	•
KEIB_DIP_02136	Calliphoridae	Calliphorinae	*Calliphora*	*subalpina*	Kabansky District, Buryatia, Russia	51.40	105.07	PQ593982	•
KEIB_DIP_02137	Calliphoridae	Calliphorinae	*Calliphora*	*subalpina* *	Kabansky District, Buryatia, Russia	51.33	104.68	Appendix 1	•
KEIB_DIP_02138	Calliphoridae	Calliphorinae	*Calliphora*	*terraenovae*	Lind, Washington, USA	46.97	-118.61	PQ594006	•
KEIB_DIP_02140	Calliphoridae	Calliphorinae	*Calliphora*	*uralensis* *	Suwalki Landscape Park, Gmina Jeleniewo, Poland	54.22	22.81	PQ594004	•
KEIB_DIP_02142	Calliphoridae	Calliphorinae	*Calliphora*	*vicina*	Kerman, Kerman Province, Iran	30.19	57.43	PQ593983	•
KEIB_DIP_02143	Calliphoridae	Calliphorinae	*Calliphora*	*varifrons*	Talyuberlup, Stirling Range National Park WA 6338, Australia	-34.41	117.95	PQ594010	•
KEIB_DIP_02145	Calliphoridae	Calliphorinae	*Calliphora*	*zaidamensis* *	Lake Teletskoye, Altai Republic, Russia	51.78	87.25	PQ594005	•
KEIB_DIP_02146	Calliphoridae	Calliphorinae	*Calliphora*	*teraramma*	Bale Mountains NP, Sanetti Plateau II, Shedem, Ethiopia	6.84	39.89	PQ593984	•
KEIB_DIP_02147	Calliphoridae	Calliphorinae	*Calliphora*	*mesay*	Bale Mountains NP, Sanetti Plateau I, Fasile Angeso, Ethiopia	6.90	39.91	PQ593985	•
KEIB_DIP_02148	Calliphoridae	Calliphorinae	*Calliphora*	*mesay*	Bale Mountains NP, Sanetti Plateau I, Fasile Angeso, Ethiopia	6.90	39.91	PQ593986	•
KEIB_DIP_02150	Calliphoridae	Calliphorinae	*Calliphora*	*teraramma*	Bale Mountains NP, Sanetti Plateau II, Shedem, Ethiopia	6.84	39.89	PQ593987	•

KEIB_DIP_02151	Calliphoridae	Calliphorinae	*Calliphora*	*teraramma*	Bale Mountains NP, Dinsho, Ethiopia	7.10	39.76	PQ593988	•
KEIB_DIP_01527	Calliphoridae	Calliphorinae	*Calliphora*	*vicina*	Święta Katarzyna, Poland	50.90	20.88	PQ593989	•
KEIB_DIP_01540	Calliphoridae	Calliphorinae	*Calliphora*	*vomitoria*	Gmina Nowa Słupia, Jeleniów, Poland	50.83	21.10	PQ593990	•
KEIB_DIP_01711	Calliphoridae	Calliphorinae	*Calliphora*	*latifrons*	Short Canyon, Sierra Sands Unified School District, CA, USA	35.70	-117.88	PQ593991	•
KEIB_DIP_01714	Calliphoridae	Calliphorinae	*Calliphora*	*triseta* *	Orosi, Cartago Province, Paraíso, Costa Rica	9.56	-83.69	Appendix 1	•
KEIB_DIP_01712	Calliphoridae	Calliphorinae	*Calliphora*	*irazuana*	Orosi, Cartago Province, Paraíso, Costa Rica	9.56	-83.69	PQ593992	•
KEIB_DIP_01672	Calliphoridae	Calliphorinae	*Calliphora*	*uralensis*	Priuralsky District, Yamalo-Nenets Autonomous Okrug, Russia	66.8	65.8	PQ593993	•
KEIB_DIP_01571	Calliphoridae	Calliphorinae	*Calliphora*	*loewi*	Gmina Janów, Siedlec, Poland	50.69	19.41	PQ594008	•
KEIB_DIP_02015	Calliphoridae	Calliphorinae	*Calliphora*	*albifrontalis*	Talyuberlup, Stirling Range National Park WA 6338, Australia	-34.41	117.95	PQ593994	•
KEIB_DIP_02018	Calliphoridae	Calliphorinae	*Calliphora*	*rohdendorfi*	Przełęcz Łaszczowa, Bardo, Poland	50.47	16.73	PQ593995	•
KEIB_DIP_02026	Calliphoridae	Calliphorinae	*Calliphora*	*ochracea*	Seven Mile Beach, Berry NSW 2535, Australia	-34.79	150.77	PQ593996	•
KEIB_DIP_02027	Calliphoridae	Calliphorinae	*Calliphora*	*dubia*	Talyuberlup, Stirling Range National Park WA 6338, Australia	-34.41	117.95	PQ593997	•
KEIB_DIP_02029	Calliphoridae	Calliphorinae	*Calliphora*	*quadrimaculata*	Mount Burns, Southland, New Zealand	-45.73	167.36	PQ593998	•
KEIB_DIP_02030	Calliphoridae	Calliphorinae	*Xenocalliphora*	*neozelandica*	Mount Burns, Southland, New Zealand	-45.73	167.36	PQ593999	•
KEIB_DIP_02032	Calliphoridae	Calliphorinae	*Calliphora*	*hilli*	Mount Keira, Wollongong, New South Wales, Australia	-34.40	150.85	PQ594000	•

KEIB_DIP_02033	Calliphoridae	Calliphorinae	*Calliphora*	*sternalis*	Forest Path Track, Royal National Park, New South Wales, Australia	-34.14	151.03	PQ594001	•
KEIB_DIP_01463	Calliphoridae	Calliphorinae	*Calliphora*	*subalpina*	89143 Blaubeuren, Germany	48.43	9.72	PQ594009	•
KEIB_DIP_02043	Calliphoridae	Calliphorinae	*Calliphora*	*croceipalpis*	Entoto Park, Addis Ababa, Ethiopia	9.08	38.73	PQ594002	•
KEIB_DIP_02085	Calliphoridae	Calliphorinae	*Onesia*	*zumpti*	71706 Markgröningen, Germany	48.90	9.06	PQ594003	•
KEIB_DIP_01557	Calliphoridae	Calliphorinae	*Onesia*	*floralis*	Nature Reserve "Zbocza Plutowskie", Chełmno, Poland	53.30	18.37	PQ635404	•
KEIB_DIP_01619	Calliphoridae	Calliphorinae	*Onesia*	*accepta*	Australian Capital Territory, Bruce Ridge National Reserve, Canberra, Australia	-35.25	149.10	PQ635405	•
KEIB_DIP_02016	Calliphoridae	Calliphorinae	*Melinda*	*viridicyanea*	Zatwarnica, Poland	49.22	22.55	PQ635406	•
KEIB_DIP_02036	Calliphoridae	Calliphorinae	*Onesia*	*tibialis*	Mount Keira, Wollongong, New South Wales, Australia	-34.40	150.85	PQ635407	•
KEIB_DIP_02037	Calliphoridae	Calliphorinae	*Onesia*	*minor*	Kambah Rock Pools, Kambah, Australian Capital Territory, Australia	-35.39	149.00	PQ635408	•
KEIB_DIP_02038	Calliphoridae	Calliphorinae	*Onesia*	*austriaca*	"Góry Pieprzowe" Nature Reserve, Sandomierz, Poland	50.68	21.79	PQ635409	•
KEIB_DIP_02039	Calliphoridae	Calliphorinae	*Onesia*	*floralis*	Natural Reserve "Borek", Bnin, Poland	53.11	17.39	PQ635410	•
KEIB_DIP_02091	Calliphoridae	Calliphorinae	*Bellardia*	*bayeri*	Tehran, Kan River Valley, Tehran, Tehran Province, Iran	35.73	51.27	PQ635411	•
KEIB_DIP_02093	Calliphoridae	Calliphorinae	*Bellardia*	*pubicornis*	Valliste, Bergsjöedet, Sweden	63.67	13.21	PQ635412	•
KEIB_DIP_02095	Calliphoridae	Calliphorinae	*Bellardia*	*siciliensis*	Ayn Darahim, Tunisia	36.78	8.68	PQ635413	•
KEIB_DIP_02096	Calliphoridae	Calliphorinae	*Bellardia*	*vespillo* *	Starogród, Rezerwat przyrody Zbocza Płutowskie, Poland	53.30	18.37	Appendix 1	•
KEIB_DIP_01528	Calliphoridae	Calliphorinae	*Bellardia*	*vulgaris*	Święta Katarzyna, Poland	50.90	20.88	PQ664927	•
KEIB_DIP_02013	Calliphoridae	Calliphorinae	*Bellardia*	*viarum*	Gmina Janów, Siedlec, Poland	50.69	19.41	PQ664928	•
KEIB_DIP_02014	Calliphoridae	Calliphorinae	*Bellardia*	*pandia*	Gmina Janów, Siedlec, Poland	50.69	19.41	PQ664929	•

KEIB_DIP_02072	Calliphoridae	Calliphorinae	*Cyanus*	*elongata*	Malheur Wildlife Station, Princeton, OR, United States	43.27	-118.80	PQ664930	•
KEIB_DIP_02074	Calliphoridae	Calliphorinae	*Cynomya*	*cadaverina*	Sprague Ave, Spokane Valley, WA 99206, USA	47.65	-117.28	PQ664931	•
KEIB_DIP_02028	Calliphoridae	Calliphorinae	*Cynomya*	*mortuorum*	Płock, Poland	52.56	19.67	PQ664986	•
KEIB_DIP_01969	Calliphoridae	Calliphorinae	*Pericallimyia*	sp.	Bale Mountains NP, 4P8Q+965 Dinshu, Ethiopia	7.11	39.73	PQ665286	•
KEIB_DIP_02088	Calliphoridae	Calliphorinae	*Polleniopsis*	*mongolica* *	Mendeleevo, Moscow Oblast, Russia	56.03	37.23	Appendix 1	•
KEIB_DIP_02089	Calliphoridae	Calliphorinae	*Polleniopsis*	*zaitzevi* *	Khakghala, Nuwara Eliya, Sri Lanka	6.95	80.79	Appendix 1	•
KEIB_DIP_02076	Calliphoridae	Calliphorinae	*Cynomyiomima*	*stackelbergi* *	Delger, Govi-Altai, Mongolia	46.35	97.35	Appendix 1	•
KEIB_DIP_02020	Calliphoridae	Calliphorinae	*Melinda*	*viridicyanea*	Kamieniołom Kantyna, Poland	50.89	16.69	PQ664953	•
KEIB_DIP_02090	Calliphoridae	Calliphorinae	*Tricycleopsis*	*paradoxa* *	Nagasaki, Japan	32.75	129.87	Appendix 1	•
KEIB_DIP_02023	Calliphoridae	Calliphorinae	*Paradichosia*	*okazaki*	Partizansky District, Primorsky Krai, Volchanka, Russia	42.90	132.72	PQ664954	•
KEIB_DIP_01822	Calliphoridae	Calliphorinae	*Eggisops*	*pecchiolii*	Blaubeuren, Germany	48.43	9.72	PQ664955	•
KEIB_DIP_02041	Calliphoridae	Calliphorinae	*Sarconesia*	*magellanica*	Cafayate Department, Salta Province, Argentina	-26.07	-65.89	PQ649436	•
KEIB_DIP_01987	Calliphoridae	Luciliinae	*Hemipyrellia*	*fernandica*	Shecha, Arba Minch, Nechisar NP, Ethiopia	6.01	37.55	PQ649437	•
KEIB_DIP_01636	Calliphoridae	Luciliinae	*Lucilia*	*porphyrina*	Cascades Walking Track, Macquarie Pass NSW, Australia	-34.56	150.67	PQ649438	•
KEIB_DIP_02007	Calliphoridae	Luciliinae	*Lucilia*	*eximia*	Chutes Fourgassier, Roura, French Guiana	4.62	-52.30	PQ649439	•
KEIB_DIP_02268	Calliphoridae	Chrysomyinae	*Cochliomyia*	*minima*	University of Puerto Rico, Río Piedras, San Juan, Puerto Rico	18.40	-66.05	PQ649440	•
KEIB_DIP_02272	Calliphoridae	Chrysomyinae	*Compsomyiops*	*fulvicrura*	Alfonsin Raul Dr 212, Río Gallegos, Santa Cruz, Argentina	-51.62	-69.22	PQ664987	•
KEIB_DIP_01559	Calliphoridae	Chrysomyinae	*Chrysomya*	*albiceps*	Gmina Janów, Siedlec, Poland	50.69	19.41	PQ665287	•
KEIB_DIP_02009	Calliphoridae	Ameniinae	*Amenia*	*imperialis*	Berrara Creek, Mondayong, NSW, Australia	-35.20	150.53	PQ665281	•

KEIB_DIP_02012	Calliphoridae	Ameniinae	*Paramenia*	*semiauriceps*	Minnamurra Rainforest, Minnamurra Falls Rd, Jamberoo NSW, Australia	-34.63	150.72	PQ665282	•
KEIB_DIP_02017	Calliphoridae	Ameniinae	*Eurychaeta*	*palpalis*	Zatwarnica, Poland	49.22	22.55	PQ665283	•
KEIB_DIP_01983	Calliphoridae	Bengaliinae	*Tricyclea*	sp. 2	Arba Minch, Nechisar NP, Ethiopia	6.01	37.55	PQ654328	•
KEIB_DIP_01980	Calliphoridae	Bengaliinae	*Cordylobia*	*anthropophaga*	Sikela Street, Arba Minch, Ethiopia	6.03	37.55	PQ654329	•
KEIB_DIP_01981	Calliphoridae	Bengaliinae	*Bengalia*	*seniorwhitei*	Arba Minch, Nechisar NP, Ethiopia	6.01	37.55	PQ654330	•
KEIB_DIP_01707	Calliphoridae	Phumosinae	*Phumosia*	*promittens*	Genting Highlands, Pahang, Malaysia	3.39	101.78	PQ654331	•
KEIB_DIP_01692	Calliphoridae	Rhiniinae	*Rhyncomya*	*zumpti*	Trans-Kalahari Highway, Wilhelmstal, Namibia	-21.90	16.38	PQ654332	•
KEIB_DIP_01708	Ulurumyiidae	….	*Ulurumyia*	*macalpinei*	Blue Mountains, Megalong Valley, Australia	-33.65	150.27	PQ654165	•
KEIB_DIP_02000	Mesembrinellidae	Mesembrinellinae	*Mesembrinella*	*benoisti*	Amazone Nature Lodge, Camp Caimans, French Guiana	4.55	-52.20	PQ654166	•
KP872701	Calliphoridae	Calliphorinae	*Calliphora*	*grahami*	Changsha, Hunan, China	28.26	112.98	KP872701	GenBank
OU696535	Calliphoridae	Calliphorinae	*Bellardia*	*pandia*	Berkshire, Wytham woods, Oxford, United Kingdom	51.76	-1.33	OU696535	GenBank
OX493271	Calliphoridae	Calliphorinae	*Bellardia*	*bayeri*	Berkshire, Wytham woods, Oxford, United Kingdom	51.76	-1.32	OX493271	GenBank
OY288238	Calliphoridae	Calliphorinae	*Calliphora*	*vicina*	Wigmore Park, Percival Way, Wigmore, Luton, East of England, England, United Kingdom	51.88	-0.36	OY288238	GenBank
MT017724	Calliphoridae	Calliphorinae	*Calliphora*	*nigribarbis*	Jining, Shandong Province,China	35.40	116.57	MT017724	GenBank
MT017707	Calliphoridae	Calliphorinae	*Calliphora*	*sinensis*	Jining, Shandong Province,China	35.40	116.57	MT017707	GenBank

MT017722	Calliphoridae	Calliphorinae	*Calliphora*	*uralensis*	Jining, Shandong Province,China	35.40	116.57	MT017722	GenBank
MT628574	Calliphoridae	Calliphorinae	*Cynomya*	*mortuorum*	Copenhagen, Denmark	55.67	12.56	MT628574	GenBank
MN131052	Calliphoridae	Calliphorinae	*Polleniopsis*	*mongolica*	Changsha, Hunan, China	28.25	112.95	MN131052	GenBank
MK893471	Calliphoridae	Calliphorinae	*Calliphora*	*calliphoroides*	Mount Bukhan, Seoul, South Korea	38.08	127.02	MK893471	GenBank
MK591038	Calliphoridae	Bengalinae	*Bengalia*	sp.	Jianfengling National Forest Park, Hainan province, China	18.70	108.92	MK591038	GenBank
OW121746	Calliphoridae	Rhiniinae	*Stomorhina*	*lunata*	Oxfordshire, Hartslock Reserve, United Kingdom	51.51	-1.11	OW121746	GenBank
OR497843	Calliphoridae	Rhiniinae	*Isomyia*	*nebulosa*	Cangyuan, Lincang, Yunnan, China	23.90	99.23	OR497843	GenBank
OW799239	Calliphoridae	Rhinophorinae	*Phyto*	*melanocephala*	Hartslock, England, United Kingdom	51.51	-1.11	OW799239	GenBank
OY757193	Calliphoridae	Rhinophorinae	*Melanophora*	*roralis*	Gerrans Bay, England, United Kingdom	50.2	-4.94	OY757193	GenBank
KT272778.1	Mesembrinellidae	Mesembrinellidae	*Mesembrinella*	sp. *	Salesópolis, SP, Brazil	-23.52	-45.84	KT272778.1	GenBank
KEIB_DIP_02118	Calliphoridae	Calliphorinae	*Calliphora*	*loewi*	Kabansky District, Buryatia, Russia	51.38	105.17	PQ654167	•
KEIB_DIP_02128	Calliphoridae	Calliphorinae	*Calliphora*	*peruviana* *	Colombia	7.28	-76.09	Appendix 1	•
KEIB_DIP_01594	Calliphoridae	Calliphorinae	*Bellardia*	*stricta*	Podgórz, 87-100 Toruń, Poland	52.97	18.58	PQ654168	•
KEIB_DIP_01541	Calliphoridae	Rhinophorinae	*Rhinomorinia*	*sarcophagina*	Jeleniów, Poland	50.83	21.10	PQ654169	•
KEIB_DIP_02069	Sarcophagidae	…	*Nephochaetopteryx*	*orbitalis*	Amazone Nature Lodge, French Guiana	4.55	-52.20	PQ654170	•
MT584151	Calliphoridae	Calliphorinae	*Calliphora*	*vomitoria*	Copenhagen, Denmark	55.67	12.56	MT584151	GenBank
KT936147	Calliphoridae	Calliphorinae	*Calliphora*	*chinghaiensis*	Shangri-la, Yunnan Province, China	27.72	99.94	KT936147	GenBank

Table S2. 114 specimens of Calliphoridae and outgroups used for mitochondrial characterization and phylogeny reconstruction, along with GenBank accession numbers, total mitogenome length and base composition. A "•" and "*" represents the specimens sequenced in this study and species with partial mitochondrial genome, respectively. For partial mitogenomes, information on missing genes is in Appendix 2. The table has been reformatted for consistency; the original version is available online at: https://doi.org/10.1016/j.ijbiomac.2025.147063.

							Base composition (%)						
ID	**Family**	**Subfamily**	**Genera**	**Species**	**Total length (bp)**	**GenBank Accession**	**T**	**C**	**A**	**G**	**AT-skew**	**GC-skew**	**Included in**
KEIB_DIP_02070	Calliphoridae	Calliphorinae	*Calliphora*	sp. n. 3	15474	PQ593971	37.5	13.8	39	9.7	0.0196	-0.1745	Phylogeny & Mtgenome Characterization
KEIB_DIP_02099	Calliphoridae	Calliphorinae	*Calliphora*	*alaskensis*	14908	PQ593972	37.7	13.7	38.8	9.7	0.0144	-0.1709	-do-
KEIB_DIP_02101	Calliphoridae	Calliphorinae	*Calliphora*	*augur* *	NA	Appendix 1							-do-
KEIB_DIP_02102	Calliphoridae	Calliphorinae	*Calliphora*	*calliphoroides*	15152	PQ593973	37.4	13.9	38.7	9.9	0.0171	-0.1681	-do-
KEIB_DIP_02104	Calliphoridae	Calliphorinae	*Calliphora*	*coloradensis*	15964	PQ593974	39.6	9.2	38.3	12.8	-0.0167	0.1636	-do-
KEIB_DIP_02109	Calliphoridae	Calliphorinae	*Calliphora*	*genarum*	15387	PQ593975	39.4	9.4	37.9	13.3	-0.0194	0.1718	-do-
KEIB_DIP_02110	Calliphoridae	Calliphorinae	*Calliphora*	*grahami*	14909	PQ593976	39.1	9.6	37.8	13.5	-0.0169	0.1688	-do-
KEIB_DIP_02112	Calliphoridae	Calliphorinae	*Calliphora*	*hilli*	16443	PQ593977	39.6	9.5	37.6	13.4	-0.0259	0.1703	-do-
KEIB_DIP_02113	Calliphoridae	Calliphorinae	*Calliphora*	*himalayana*	14920	PQ593978	38.9	9.6	37.6	14	-0.017	0.1864	-do-
KEIB_DIP_02115	Calliphoridae	Calliphorinae	*Calliphora*	*livida* *	NA	Appendix 1							-do-
KEIB_DIP_02116	Calliphoridae	Calliphorinae	*Calliphora*	*livida* *	NA	Appendix 1							-do-
KEIB_DIP_02121	Calliphoridae	Calliphorinae	*Calliphora*	*montana* *	NA	Appendix 1							-do-
KEIB_DIP_02122	Calliphoridae	Calliphorinae	*Calliphora*	*calliphoroides*	14907	PQ594007	38.6	10	37.4	14	-0.0158	0.1667	-do-
KEIB_DIP_02123	Calliphoridae	Calliphorinae	*Calliphora*	*nigribarbis* *	NA	Appendix 1							-do-
KEIB_DIP_02124	Calliphoridae	Calliphorinae	*Calliphora*	*nigrithorax*	15801	PQ593979	37.7	13.3	39.6	9.4	0.0246	-0.1718	-do-
KEIB_DIP_02130	Calliphoridae	Calliphorinae	*Calliphora*	*rohdendorfi*	15285	PQ593980	37.7	13.6	39.1	9.6	0.0182	-0.1724	-do-
KEIB_DIP_02131	Calliphoridae	Calliphorinae	*Calliphora*	splendens *	NA	Appendix 1							-do-

KEIB_DIP_02132	Calliphoridae	Calliphorinae	*Calliphora*	*stelviana**	NA	Appendix 1							-do-
KEIB_DIP_02135	Calliphoridae	Calliphorinae	*Calliphora*	*subalpina*	14907	PQ593981	38.9	9.7	37.7	13.7	-0.0157	0.1709	-do-
KEIB_DIP_02136	Calliphoridae	Calliphorinae	*Calliphora*	*subalpina*	15199	PQ593982	39	9.7	37.8	13.6	-0.0156	0.1674	-do-
KEIB_DIP_02137	Calliphoridae	Calliphorinae	*Calliphora*	*subalpina* *	NA	Appendix 1							-do-
KEIB_DIP_02138	Calliphoridae	Calliphorinae	*Calliphora*	*terraenovae*	14907	PQ594006	38.8	9.8	37.8	13.5	-0.0131	0.1588	-do-
KEIB_DIP_02140	Calliphoridae	Calliphorinae	*Calliphora*	*uralensis* *	15029	PQ594004	39.2	9.6	37.9	13.3	-0.0169	0.1616	-do-
KEIB_DIP_02142	Calliphoridae	Calliphorinae	*Calliphora*	*vicina*	14920	PQ593983	38.8	9.8	37.8	13.6	-0.0131	0.1624	-do-
KEIB_DIP_02143	Calliphoridae	Calliphorinae	*Calliphora*	*varifrons*	16356	PQ594010	38.5	11	36.8	13.7	-0.0226	0.1093	-do-
KEIB_DIP_02145	Calliphoridae	Calliphorinae	*Calliphora*	*zaidamensis* *	13062	PQ594005	37.3	13.8	38.6	10.3	0.0171	-0.1452	-do-
KEIB_DIP_02146	Calliphoridae	Calliphorinae	*Calliphora*	*teraramma*	15779	PQ593984	39.3	9.3	38.3	13	-0.0129	0.1659	-do-
KEIB_DIP_02147	Calliphoridae	Calliphorinae	*Calliphora*	*mesay*	14928	PQ593985	37.8	13.6	38.8	9.8	0.0131	-0.1624	-do-
KEIB_DIP_02148	Calliphoridae	Calliphorinae	*Calliphora*	*mesay*	14928	PQ593986	37.8	13.6	38.8	9.8	0.0131	-0.1624	-do-
KEIB_DIP_02150	Calliphoridae	Calliphorinae	*Calliphora*	*teraramma*	15776	PQ593987	38.3	13	39.3	9.3	0.0129	-0.1659	-do-
KEIB_DIP_02151	Calliphoridae	Calliphorinae	*Calliphora*	*teraramma*	15779	PQ593988	38.3	13	39.3	9.3	0.0129	-0.1659	-do-
KEIB_DIP_01527	Calliphoridae	Calliphorinae	*Calliphora*	*vicina*	16386	PQ593989	38.1	13	39.5	9.3	0.018	-0.1659	-do-
KEIB_DIP_01540	Calliphoridae	Calliphorinae	*Calliphora*	*vomitoria*	16138	PQ593990	38.1	13.1	39.5	9.3	0.018	-0.1696	-do-
KEIB_DIP_01711	Calliphoridae	Calliphorinae	*Calliphora*	*latifrons*	15887	PQ593991	37.6	13.7	39	9.7	0.0183	-0.1709	-do-
KEIB_DIP_01714	Calliphoridae	Calliphorinae	*Calliphora*	*triseta* *	NA	Appendix 1							-do-
KEIB_DIP_01712	Calliphoridae	Calliphorinae	*Calliphora*	*irazuana*	14908	PQ593992	37.6	13.7	38.7	10	0.0144	-0.1561	-do-
KEIB_DIP_01672	Calliphoridae	Calliphorinae	*Calliphora*	*uralensis*	14909	PQ593993	37.8	13.5	38.8	9.8	0.0131	-0.1588	-do-
KEIB_DIP_01571	Calliphoridae	Calliphorinae	*Calliphora*	*loewi*	14908	PQ594008	38.7	9.8	37.7	13.8	-0.0131	0.1695	-do-
KEIB_DIP_02015	Calliphoridae	Calliphorinae	*Calliphora*	*albifrontalis*	14911	PQ593994	37.3	14	38.8	9.9	0.0197	-0.1715	-do-
KEIB_DIP_02018	Calliphoridae	Calliphorinae	*Calliphora*	*rohdendorfi*	15024	PQ593995	37.5	13.9	38.9	9.8	0.0183	-0.173	-do-
KEIB_DIP_02026	Calliphoridae	Calliphorinae	*Calliphora*	*ochracea*	16554	PQ593996	38.2	12.8	39.8	9.1	0.0205	-0.1689	-do-
KEIB_DIP_02027	Calliphoridae	Calliphorinae	*Calliphora*	*dubia*	14909	PQ593997	37.3	13.6	39.2	9.9	0.0248	-0.1574	-do-
KEIB_DIP_02029	Calliphoridae	Calliphorinae	*Calliphora*	*quadrimaculata*	15704	PQ593998	38	13.3	39.3	9.4	0.0168	-0.1718	-do-
KEIB_DIP_02030	Calliphoridae	Calliphorinae	*Xenocalliphora*	*neozelandica*	15193	PQ593999	39.4	9.6	37.6	13.4	-0.0234	0.1652	-do-
KEIB_DIP_02032	Calliphoridae	Calliphorinae	*Calliphora*	*hilli*	16414	PQ594000	39.6	9.4	37.6	13.4	-0.0259	0.1754	-do-
KEIB_DIP_02033	Calliphoridae	Calliphorinae	*Calliphora*	*sternalis*	14921	PQ594001	37	14.1	39.3	9.6	0.0301	-0.1899	-do-
KEIB_DIP_01463	Calliphoridae	Calliphorinae	*Calliphora*	*subalpina*	14905	PQ594009	38.8	9.7	37.8	13.7	-0.0131	0.1709	-do-
KEIB_DIP_02043	Calliphoridae	Calliphorinae	*Calliphora*	*croceipalpis*	14908	PQ594002	36.9	14.5	38.5	10.1	0.0212	-0.1789	-do-

KEIB_DIP_02085	Calliphoridae	Calliphorinae	*Onesia*	*zumpti*	15029	PQ594003	38.5	12.3	40.1	9.1	0.0204	-0.1495	-do-
KEIB_DIP_01557	Calliphoridae	Calliphorinae	*Onesia*	*floralis*	15027	PQ635404	39.7	9.3	38.2	12.7	-0.0193	0.1545	-do-
KEIB_DIP_01619	Calliphoridae	Calliphorinae	*Onesia*	*accepta*	15086	PQ635405	39.6	9.4	37.9	13.2	-0.0219	0.1681	-do-
KEIB_DIP_02016	Calliphoridae	Calliphorinae	*Melinda*	*viridicyanea*	15123	PQ635406	38.7	12.2	40.2	8.9	0.019	-0.1564	-do-
KEIB_DIP_02036	Calliphoridae	Calliphorinae	*Onesia*	*tibialis*	16942	PQ635407	40.4	9	38.5	12.1	-0.0241	0.1469	-do-
KEIB_DIP_02037	Calliphoridae	Calliphorinae	*Onesia*	*minor*	14916	PQ635408	39	9.8	36.5	14.6	-0.0331	0.1967	-do-
KEIB_DIP_02038	Calliphoridae	Calliphorinae	*Onesia*	*austriaca*	14904	PQ635409	39.8	9.4	38.2	12.7	-0.0205	0.1493	-do-
KEIB_DIP_02039	Calliphoridae	Calliphorinae	*Onesia*	*floralis*	14997	PQ635410	38.2	12.7	39.8	9.3	0.0205	-0.1545	-do-
KEIB_DIP_02091	Calliphoridae	Calliphorinae	*Bellardia*	*bayeri*	14995	PQ635411	37	13.9	39.9	9.1	0.0377	-0.2087	-do-
KEIB_DIP_02093	Calliphoridae	Calliphorinae	*Bellardia*	*pubicornis*	15116	PQ635412	39.9	9.2	37.2	13.7	-0.035	0.1965	-do-
KEIB_DIP_02095	Calliphoridae	Calliphorinae	*Bellardia*	*siciliensis*	15888	PQ635413	37.6	13.2	40.3	8.9	0.0347	-0.1946	-do-
KEIB_DIP_02096	Calliphoridae	Calliphorinae	*Bellardia*	*vespillo* *	NA	Appendix 1							-do-
KEIB_DIP_01528	Calliphoridae	Calliphorinae	*Bellardia*	*vulgaris*	16216	PQ664927	40	9.2	37.8	13	-0.0283	0.1712	-do-
KEIB_DIP_02013	Calliphoridae	Calliphorinae	*Bellardia*	*viarum*	15090	PQ664928	39.9	9.1	37.5	13.5	-0.031	0.1947	-do-
KEIB_DIP_02014	Calliphoridae	Calliphorinae	*Bellardia*	*pandia*	15108	PQ664929	39.9	9.2	37	13.9	-0.0377	0.2035	-do-
KEIB_DIP_02072	Calliphoridae	Calliphorinae	*Cyanus*	*elongata*	14897	PQ664930	37.3	14	38.9	9.8	0.021	-0.1765	-do-
KEIB_DIP_02074	Calliphoridae	Calliphorinae	*Cynomya*	*cadaverina*	14904	PQ664931	37.4	14	38.5	10.1	0.0145	-0.1618	-do-
KEIB_DIP_02028	Calliphoridae	Calliphorinae	*Cynomya*	*mortuorum*	14903	PQ664986	37.4	14.1	38.4	10.1	0.0132	-0.1653	-do-
KEIB_DIP_01969	Calliphoridae	Calliphorinae	*Pericallimyia*	sp.	15619	PQ665286	40.3	8.9	38.1	12.8	-0.0281	0.1797	-do-
KEIB_DIP_02088	Calliphoridae	Calliphorinae	*Polleniopsis*	*mongolica* *	NA	Appendix 1							-do-
KEIB_DIP_02089	Calliphoridae	Calliphorinae	*Polleniopsis*	*zaitzevi* *	NA	Appendix 1							-do-
KEIB_DIP_02076	Calliphoridae	Calliphorinae	*Cynomyiomima*	*stackelbergi* *	NA	Appendix 1							-do-
KEIB_DIP_02020	Calliphoridae	Calliphorinae	*Melinda*	*viridicyanea*	15052	PQ664953	38.6	12.3	40.2	9	0.0203	-0.1549	-do-
KEIB_DIP_02090	Calliphoridae	Calliphorinae	*Tricycleopsis*	*paradoxa* *	NA	Appendix 1							-do-
KEIB_DIP_02023	Calliphoridae	Calliphorinae	*Paradichosia*	*okazaki*	15293	PQ664954	40.2	8.8	38.8	12.1	-0.0177	0.1579	-do-
KEIB_DIP_01822	Calliphoridae	Calliphorinae	*Eggisops*	*pecchiolii*	15698	PQ664955	37.4	13.4	40.5	8.7	0.0398	-0.2127	-do-
KEIB_DIP_02041	Calliphoridae	Calliphorinae	*Sarconesia*	*magellanica*	15079	PQ649436	37.7	13.2	39.3	9.7	0.0208	-0.1528	-do-
KEIB_DIP_01987	Calliphoridae	Luciliinae	*Hemipyrellia*	*fernandica*	16127	PQ649437	38	13.2	39.6	9.1	0.0206	-0.1839	-do-
KEIB_DIP_01636	Calliphoridae	Luciliinae	*Lucilia*	*porphyrina*	15874	PQ649438	37.2	14.2	39	9.6	0.0236	-0.1933	-do-
KEIB_DIP_02007	Calliphoridae	Luciliinae	*Lucilia*	*eximia*	15990	PQ649439	37.8	13.8	39.1	9.3	0.0169	-0.1948	-do-
KEIB_DIP_02268	Calliphoridae	Chrysomyinae	*Cochliomyia*	*minima*	16049	PQ649440	37.9	13.3	39.2	9.5	0.0169	-0.1667	-do-

KEIB_DIP_02272	Calliphoridae	Chrysomyinae	*Compsomyiops*	*fulvicrura*	16038	PQ664987	39.2	9.5	38	13.3	-0.0155	0.1667	-do-
KEIB_DIP_01559	Calliphoridae	Chrysomyinae	*Chrysomya*	*albiceps*	14921	PQ665287	37.4	13.6	39.5	9.4	0.0273	-0.1826	-do-
KEIB_DIP_02009	Calliphoridae	Ameniinae	*Amenia*	*imperialis*	16198	PQ665281	40.3	8.7	37.8	13.3	-0.032	0.2091	-do-
KEIB_DIP_02012	Calliphoridae	Ameniinae	*Paramenia*	*semiauriceps*	17267	PQ665282	37.6	12.7	41.3	8.3	0.0469	-0.2095	-do-
KEIB_DIP_02017	Calliphoridae	Ameniinae	*Eurychaeta*	*palpalis*	16593	PQ665283	38.6	12.6	39.9	8.8	0.0166	-0.1776	-do-
KEIB_DIP_01983	Calliphoridae	Bengaliinae	*Tricyclea*	sp. 2	14961	PQ654328	37.5	13.2	40	9.4	0.0323	-0.1681	-do-
KEIB_DIP_01980	Calliphoridae	Bengaliinae	*Cordylobia*	*anthropophaga*	15476	PQ654329	37.1	13.7	40.2	9	0.0401	-0.207	-do-
KEIB_DIP_01981	Calliphoridae	Bengaliinae	*Bengalia*	*seniorwhitei*	15907	PQ654330	37.7	13.5	39.8	9.1	0.0271	-0.1947	-do-
KEIB_DIP_01707	Calliphoridae	Phumosinae	*Phumosia*	*promittens*	16562	PQ654331	37.3	13.5	40.1	9.1	0.0362	-0.1947	-do-
KEIB_DIP_01692	Calliphoridae	Rhiniinae	*Rhyncomya*	*zumpti*	16348	PQ654332	37.6	12.6	40.8	9	0.0408	-0.1667	-do-
KEIB_DIP_01708	Ulurumyiidae	….	*Ulurumyia*	*macalpinei*	15140	PQ654165	39.1	9.6	37.2	14.2	-0.0249	0.1933	-do-
KEIB_DIP_02000	Mesembrinellidae	Mesembrinellinae	*Mesembrinella*	*benoisti*	15320	PQ654166	39.8	8.9	39	12.3	-0.0102	0.1604	-do-
KP872701	Calliphoridae	Calliphorinae	*Calliphora*	*grahami*	14903	KP872701	37.6	13.7	39.1	9.6	0.0196	-0.176	-do-
OU696535	Calliphoridae	Calliphorinae	*Bellardia*	*pandia*	21636	OU696535	42.1	7.8	39	11.2	-0.0382	0.1789	-do-
OX493271	Calliphoridae	Calliphorinae	*Bellardia*	*bayeri*	21015	OX493271	42	7.6	38.7	11.6	-0.0409	0.2083	-do-
OY288238	Calliphoridae	Calliphorinae	*Calliphora*	*vicina*	16716	OY288238	39.9	9.1	38.1	13	-0.0231	0.1765	-do-
MT017724	Calliphoridae	Calliphorinae	*Calliphora*	*nigribarbis*	16241	MT017724	37.9	13.2	39.6	9.3	0.0219	-0.1733	-do-
MT017707	Calliphoridae	Calliphorinae	*Calliphora*	*sinensis*	16234	MT017707	38.2	13	39.4	9.4	0.0155	-0.1607	-do-
MT017722	Calliphoridae	Calliphorinae	*Calliphora*	*uralensis*	14971	MT017722	37.8	13.5	38.9	9.8	0.0143	-0.1588	-do-
MT628574	Calliphoridae	Calliphorinae	*Cynomya*	*mortuorum*	15003	MT628574	37.4	14	38.4	10.1	0.0132	-0.1618	-do-
MN131052	Calliphoridae	Calliphorinae	*Polleniopsis*	*mongolica*	15568	MN131052	38.6	10.5	41.3	9.7	0.0338	-0.0396	-do-
MK893471	Calliphoridae	Calliphorinae	*Calliphora*	*calliphoroides*	16529	MK893471	37.9	13.3	39.3	9.5	0.0181	-0.1667	-do-
MK591038	Calliphoridae	Bengalinae	*Bengalia*	sp.	15784	MK591038	37.7	13.7	39.3	9.3	0.0208	-0.1913	-do-
OW121746	Calliphoridae	Rhiniinae	*Stomorhina*	*lunata*	16491	OW121746	40.4	8.9	38.4	12.3	-0.0254	0.1604	-do-
OR497843	Calliphoridae	Rhiniinae	*Isomyia*	*nebulosa*	16438	OR497843	38.3	12.4	40.8	8.5	0.0316	-0.1866	-do-
OW799239	Calliphoridae	Rhinophorinae	*Phyto*	*melanocephala*	16280	OW799239	41.5	8.1	38.8	11.6	-0.0336	0.1777	-do-
OY757193	Calliphoridae	Rhinophorinae	*Melanophora*	*roralis*	19441	OY757193	43.5	7	39.3	10.2	-0.0507	0.186	-do-
KT272778.1	Mesembrinellidae	Mesembrinellidae	*Mesembrinella*	sp. *	14856	KT272778.1	38.7	12.6	39.7	9	0.0128	-0.1667	-do-
KEIB_DIP_02118	Calliphoridae	Calliphorinae	*Calliphora*	*loewi*	14908	PQ654167	38.8	9.8	37.8	13.7	-0.0131	0.166	Mtgenome Characterization
KEIB_DIP_02128	Calliphoridae	Calliphorinae	*Calliphora*	*peruviana* *	NA	Appendix 1							-do-

KEIB_DIP_01594	Calliphoridae	Calliphorinae	*Bellardia*	*stricta*	14910	PQ654168	39.8	9.4	38.2	12.7	-0.0205	0.1493	-do-
KEIB_DIP_01541	Calliphoridae	Rhinophorinae	*Rhinomorinia*	*sarcophagina*	15310	PQ654169	39.8	9.2	38.3	12.6	-0.0192	0.156	-do-
KEIB_DIP_02069	Sarcophagidae	…	*Nephochaetopteryx*	*orbitalis*	16313	PQ654170	37	13.8	40	9.3	0.039	-0.1948	-do-
MT584151	Calliphoridae	Calliphorinae	*Calliphora*	*vomitoria*	15257	MT584151	37.8	13.6	39	9.6	0.0156	-0.1724	-do-
KT936147	Calliphoridae	Calliphorinae	*Calliphora*	*chinghaiensis*	15269	KT936147	37.5	13.3	39.3	10	0.0234	-0.1416	-do-

Appendix 1

			GenBank Accession												
			ATP6	**ATP8**	**COI**	**COII**	**COIII**	**CYTB**	**ND1**	**ND2**	**ND3**	**ND4**	**ND4L**	**ND5**	**ND6**
KEIB_DIP_02101	*Calliphora*	*augur* *	PQ438194	PQ438209	PQ428990	PQ438224	PQ438239	PQ438254	PQ438269	PQ438284	PQ438299	PQ438314	PQ438329	PQ438344	PQ438359
KEIB_DIP_02115	*Calliphora*	*livida* *	PQ438195	PQ438210	PQ428991	PQ438225	PQ438240	PQ438255	PQ438270	PQ438285	PQ438300	PQ438315	PQ438330	PQ438345	PQ438360
KEIB_DIP_02116	*Calliphora*	*livida* *	PQ438196	PQ438211	PQ428992	PQ438226	PQ438241	PQ438256	PQ438271	PQ438286	PQ438301	PQ438316	PQ438331	PQ438346	PQ438361
KEIB_DIP_02121	*Calliphora*	*montana**	PQ438197	PQ438212	PQ428993	PQ438227	PQ438242	PQ438257	PQ438272	PQ438287	PQ438302	PQ438317	PQ438332	PQ438347	PQ438362
KEIB_DIP_02123	*Calliphora*	*nigribarbis**	PQ438198	PQ438213	PQ428994	PQ438228	PQ438243	PQ438258	PQ438273	PQ438288	PQ438303	PQ438318	PQ438333	PQ438348	PQ438363
KEIB_DIP_02131	*Calliphora*	*splendens**	PQ438199	PQ438214	PQ428995	PQ438229	PQ438244	PQ438259	PQ438274	PQ438289	PQ438304	PQ438319	PQ438334	PQ438349	PQ438364
KEIB_DIP_02132	*Calliphora*	*stelviana**	PQ438200	PQ438215	PQ428996	PQ438230	PQ438245	PQ438260	PQ438275	PQ438290	PQ438305	PQ438320	PQ438335	PQ438350	PQ438365
KEIB_DIP_02137	*Calliphora*	*subalpina**	PQ438201	PQ438216	PQ428997	PQ438231	PQ438246	PQ438261	PQ438276	PQ438291	PQ438306	PQ438321	PQ438336	PQ438351	PQ438366
KEIB_DIP_01714	*Calliphora*	*triseta**	PQ438202	PQ438217	PQ428998	PQ438232	PQ438247	PQ438262	PQ438277	PQ438292	PQ438307	PQ438322	PQ438337	PQ438352	PQ438367
KEIB_DIP_02096	*Bellardia*	*vespillo**	PQ438203	PQ438218	PQ428999	PQ438233	PQ438248	PQ438263	PQ438278	PQ438293	PQ438308	PQ438323	PQ438338	PQ438353	PQ438368
KEIB_DIP_02088	*Polleniopsis*	*mongolica**	PQ438204	PQ438219	PQ429000	PQ438234	PQ438249	PQ438264	PQ438279	PQ438294	PQ438309	PQ438324	PQ438339	PQ438354	PQ438369
KEIB_DIP_02089	*Polleniopsis*	*zaitzevi**	PQ438205	PQ438220	PQ429001	PQ438235	PQ438250	PQ438265	PQ438280	PQ438295	PQ438310	PQ438325	PQ438340	PQ438355	PQ438370
KEIB_DIP_02076	*Cynomyiomima*	*stackelbergi**	PQ438206	PQ438221	PQ429002	PQ438236	PQ438251	PQ438266	PQ438281	PQ438296	PQ438311	PQ438326	PQ438341	PQ438356	PQ438371
KEIB_DIP_02090	*Tricycleopsis*	*paradoxa**	PQ438207	PQ438222	PQ429003	PQ438237	PQ438252	PQ438267	PQ438282	PQ438297	PQ438312	PQ438327	PQ438342	PQ438357	PQ438372
KEIB_DIP_02128	*Calliphora*	*peruviana**	PQ438208	PQ438223	PQ429004	PQ438238	PQ438253	PQ438268	PQ438283	PQ438298	PQ438313	PQ438328	PQ438343	PQ438358	PQ438373

Appendix 2							
ID	**Family**	**Subfamily**	**Genera**	**Species**	**Total length (bp)**	**GenBank Accession**	**Genes which were not obtained**
KEIB_DIP_02101	Calliphoridae	Calliphorinae	*Calliphora*	*augur* *	NA	Appendix 1	SrRNA, LrRNA, trnA, trnR, trnN, trnS1, trnE, trnF, trnV
KEIB_DIP_02115	Calliphoridae	Calliphorinae	*Calliphora*	*livida* *	NA	Appendix 1	trnI, trnQ, trnA, trnR, trnN, trnS1
KEIB_DIP_02116	Calliphoridae	Calliphorinae	*Calliphora*	*livida* *	NA	Appendix 1	trnD
KEIB_DIP_02123	Calliphoridae	Calliphorinae	*Calliphora*	*nigribarbis* *	NA	Appendix 1	SrRNA, trnA, trnR, trnN, trnS1, trnE, trnF, trnV
KEIB_DIP_02131	Calliphoridae	Calliphorinae	*Calliphora*	splendens *	NA	Appendix 1	SrRNA, LrRNA, trnI, trnQ, trnM, trnW, trnC, trnY, trnL2, trnG, trnA, trnR, trnN, trnS1, trnE, trnF, trnH, trnT, trnP, trnL1, trnV
KEIB_DIP_02132	Calliphoridae	Calliphorinae	*Calliphora*	*stelviana**	NA	Appendix 1	trnL2, trnD, trnH, trnT, trnP, trnV
KEIB_DIP_02137	Calliphoridae	Calliphorinae	*Calliphora*	*subalpina* *	NA	Appendix 1	LrRNA, trnQ, trnM, trnW, trnC, trnY, trnR, trnN, trnS1, trnE, trnF, trnH, trnS2, trnL1,
KEIB_DIP_02140	Calliphoridae	Calliphorinae	*Calliphora*	*uralensis* *	15029	PQ594004	trnI, trnQ, trnM
KEIB_DIP_02145	Calliphoridae	Calliphorinae	*Calliphora*	*zaidamensis* *	13062	PQ594005	SrRNA
KEIB_DIP_01714	Calliphoridae	Calliphorinae	*Calliphora*	*triseta* *	NA	Appendix 1	trnI, trnQ, trnM
KEIB_DIP_02091	Calliphoridae	Calliphorinae	*Bellardia*	*bayeri*	14995	PQ635411	trnF
KEIB_DIP_02096	Calliphoridae	Calliphorinae	*Bellardia*	*vespillo* *	NA	Appendix 1	trnF
KEIB_DIP_02088	Calliphoridae	Calliphorinae	*Polleniopsis*	*mongolica* *	NA	Appendix 1	SrRNA, trnV
KEIB_DIP_02089	Calliphoridae	Calliphorinae	*Polleniopsis*	*zaitzevi* *	NA	Appendix 1	SrRNA, LrRNA, trnI, trnQ, trnM, trnL2, trnN, trnS1, trnE, trnF, trnV
KEIB_DIP_02076	Calliphoridae	Calliphorinae	*Cynomyiomima*	*stackelbergi* *	NA	Appendix 1	SrRNA, LrRNA, trnI, trnQ, trnM, trnT, trnP, trnV
KEIB_DIP_02090	Calliphoridae	Calliphorinae	*Tricycleopsis*	*paradoxa* *	NA	Appendix 1	SrRNA, LrRNA, trnI, trnQ, trnM, trnW, trnC, trnY, trnL2, trnG, trnH, trnT, trnP, trnV
KT272778.1	Mesembrinellidae	Mesembrinellidae	*Mesembrinella*	sp. *	14856	KT272778.1	trnQ, trnC, trnY

Table S3. Substitution saturation of all PCGs analyzed in this study using DAMBE 7 (Xia, 2018).

No. of OTUs	Iss.obs	Iss.cSym	P-value (Iss.obs vs. Iss.cSym)	Iss.cAsym	P-value (Iss.obs vs. Iss.cAsym)
4	0.155	0.858	0	0.846	0
8	0.156	0.845	0	0.762	0
16	0.164	0.85	0	0.676	0
32	0.175	0.818	0	0.572	0

OTU, operational taxonomic unit.

Iss.cAsym, the predicted Iss value under asymmetrical tree.

Iss.cSym, the predicted Iss value under symmetrical tree.

Iss.obs, index of substitution saturation for subsets of OTUs randomly sampled from full dataset.

-value is computed by Chi-square (x2) test, two-sided.

Table S4. The optimal evolutionary models for all codon positions of PCGs (PCG123), calculated by IQ-TREE according to the AIC for ML analysis.

```
#nexus
begin sets;
  charset atp6_pos1_cytb_pos1 = 1-678\3 3844-4980\3;
  charset atp6_pos2_nd1_pos2 = 2-678\3 4982-5928\3;
  charset atp6_pos3_cox1_pos3 = 3-678\3 846-2382\3;
  charset atp8_pos1 = 679-843\3;
  charset atp8_pos2_nd2_pos2 = 680-843\3 5930-6945\3;
  charset atp8_pos3 = 681-843\3;
  charset cox1_pos1 = 844-2382\3;
  charset cox1_pos2 = 845-2382\3;
  charset cox2_pos1 = 2383-3054\3;
  charset cox2_pos2_cox3_pos2 = 2384-3054\3 3056-3843\3;
  charset cox2_pos3 = 2385-3054\3;
  charset cox3_pos1 = 3055-3843\3;
  charset cox3_pos3_cytb_pos3_nd3_pos3 = 3057-3843\3 3846-4980\3 6948-
7302\3;
  charset cytb_pos2 = 3845-4980\3;
  charset nd1_pos1_nd5_pos1 = 4981-5928\3 8959-10710\3;
  charset nd1_pos3 = 4983-5928\3;
  charset nd2_pos1 = 5929-6945\3;
  charset nd2_pos3 = 5931-6945\3;
  charset nd3_pos1 = 6946-7302\3;
  charset nd3_pos2 = 6947-7302\3;
  charset nd4_pos1 = 7303-8661\3;
  charset nd4_pos2_nd5_pos2 = 7304-8661\3 8960-10710\3;
  charset nd4_pos3 = 7305-8661\3;
  charset nd4l_pos1 = 8662-8958\3;
  charset nd4l_pos2 = 8663-8958\3;
  charset nd4l_pos3 = 8664-8958\3;
  charset nd5_pos3 = 8961-10710\3;
  charset nd6_pos1 = 10711-11232\3;
  charset nd6_pos2 = 10712-11232\3;
  charset nd6_pos3 = 10713-11232\3;
  charpartition mymodels =
    GTR+F+I+I+R5: atp6_pos1_cytb_pos1,
    TIM+F+I+I+R2: atp6_pos2_nd1_pos2,
    TIM+F+I+I+R6: atp6_pos3_cox1_pos3,
    TN+F+I+G4: atp8_pos1,
    TN+F+R3: atp8_pos2_nd2_pos2,
    TN+F+I+G4: atp8_pos3,
    TIM2+F+I+I+R4: cox1_pos1,
```

```
    TIM2+F+I+I+R3: cox1_pos2,
    TIM2+F+I+I+R3: cox2_pos1,
    GTR+F+I+I+R3: cox2_pos2_cox3_pos2,
    TN+F+I+I+R5: cox2_pos3,
    TIM2+F+I+I+R3: cox3_pos1,
    GTR+F+I+I+R6: cox3_pos3_cytb_pos3_nd3_pos3,
    TN+F+I+I+R2: cytb_pos2,
    GTR+F+I+I+R4: nd1_pos1_nd5_pos1,
    TIM+F+I+I+R6: nd1_pos3,
    GTR+F+I+I+R4: nd2_pos1,
    TIM+F+I+I+R6: nd2_pos3,
    TIM2+F+I+G4: nd3_pos1,
    TIM3+F+I+G4: nd3_pos2,
    GTR+F+I+I+R3: nd4_pos1,
    TVM+F+I+I+R3: nd4_pos2_nd5_pos2,
    GTR+F+I+I+R5: nd4_pos3,
    TIM+F+G4: nd4l_pos1,
    GTR+F+I+I+R2: nd4l_pos2,
    TN+F+I+I+R4: nd4l_pos3,
    TIM+F+I+I+R5: nd5_pos3,
    GTR+F+I+I+R4: nd6_pos1,
    TVM+F+I+I+R2: nd6_pos2,
    K3Pu+F+R5: nd6_pos3;
end;
```

Table S5. Results of Kruskal–Wallis rank sum tests assessing inter-subfamily differences in nucleotide composition and strand asymmetry across Calliphoridae mitogenomes (excluding Phumosiinae).

Gene	Feature	Chi-squared	df	P-value
Entire mitochondrial genome	A+T %	18.83	6	0.0045
	G+C %	18.73	6	0.0046
	AT-skew	17.02	6	0.0092
	GC-skew	18.87	6	0.0044
Protein-coding genes	A+T %	17.83	6	0.0066
	G+C %	17.83	6	0.0066
	AT-skew	13.33	6	0.038
	GC-skew	11.46	6	0.075

Table S6. Dunn's post hoc pairwise comparisons among Calliphoridae subfamilies for nucleotide composition and strand asymmetry metrics across entire mitogenomes and protein-coding genes (PCGs). Significant adjusted p-values (p < 0.05) are highlighted in bold.

Gene	Feature	Comparison	Z	P.unadj	P.adj
Entire mitochondrial genome	A+T %	Ameniinae - Bengaliinae	1.399	0.162	0.283
		Ameniinae - Calliphorinae	2.377	0.017	0.122
		Bengaliinae - Calliphorinae	0.644	0.519	0.779
		Ameniinae - Chrysomyinae	1.693	0.09	0.211
		Bengaliinae - Chrysomyinae	0.41	0.682	0.954
		Calliphorinae - Chrysomyinae	-0.029	0.977	0.977
		Ameniinae - Luciliinae	1.978	0.048	0.168
		Bengaliinae - Luciliinae	0.715	0.475	0.767
		Calliphorinae - Luciliinae	0.366	0.715	0.938
		Chrysomyinae - Luciliinae	0.285	0.776	0.958
		Ameniinae - Rhiniinae	-0.112	0.911	0.956
		Bengaliinae - Rhiniinae	-1.52	0.129	0.246
		Calliphorinae - Rhiniinae	-2.533	0.011	0.119
		Chrysomyinae - Rhiniinae	-1.805	0.071	0.186
		Luciliinae - Rhiniinae	-2.09	0.037	0.154
		Ameniinae - Rhinophorinae	-0.27	0.787	0.919
		Bengaliinae - Rhinophorinae	-1.688	0.091	0.192
		Calliphorinae - Rhinophorinae	-2.751	0.006	0.125
		Chrysomyinae - Rhinophorinae	-1.963	0.05	0.149
		Luciliinae - Rhinophorinae	-2.247	0.025	0.129
		Rhiniinae - Rhinophorinae	-0.157	0.875	0.967
	G+C %	Ameniinae - Bengaliinae	-1.417	0.156	0.274
		Ameniinae - Calliphorinae	-2.346	0.019	0.133
		Bengaliinae - Calliphorinae	-0.583	0.56	0.84
		Ameniinae - Chrysomyinae	-1.58	0.114	0.24
		Bengaliinae - Chrysomyinae	-0.272	0.785	0.916
		Calliphorinae - Chrysomyinae	0.155	0.877	0.921
		Ameniinae - Luciliinae	-1.962	0.05	0.174
		Bengaliinae - Luciliinae	-0.68	0.496	0.802
		Calliphorinae - Luciliinae	-0.375	0.708	0.929
		Chrysomyinae - Luciliinae	-0.382	0.703	0.984
		Ameniinae - Rhiniinae	0.142	0.887	0.887
		Bengaliinae - Rhiniinae	1.569	0.117	0.223
		Calliphorinae - Rhiniinae	2.543	0.011	0.115
		Chrysomyinae - Rhiniinae	1.722	0.085	0.198
		Luciliinae - Rhiniinae	2.104	0.035	0.148
		Ameniinae - Rhinophorinae	0.3	0.765	0.944
		Bengaliinae - Rhinophorinae	1.737	0.082	0.216
		Calliphorinae - Rhinophorinae	2.762	0.006	0.121
		Chrysomyinae - Rhinophorinae	1.88	0.06	0.18
		Luciliinae - Rhinophorinae	2.262	0.024	0.125
		Rhiniinae - Rhinophorinae	0.157	0.875	0.967
		Ameniinae - Bengaliinae	-1.558	0.119	0.313
		Ameniinae - Calliphorinae	0.421	0.674	0.786

	AT-skew	Bengaliinae - Calliphorinae	2.801	0.005	**0.053**
		Ameniinae - Chrysomyinae	-0.262	0.793	0.833
		Bengaliinae - Chrysomyinae	1.278	0.201	0.423
		Calliphorinae - Chrysomyinae	-0.784	0.433	0.65
		Ameniinae - Luciliinae	-0.801	0.423	0.684
		Bengaliinae - Luciliinae	0.702	0.483	0.676
		Calliphorinae - Luciliinae	-1.531	0.126	0.293
		Chrysomyinae - Luciliinae	-0.539	0.59	0.729
		Ameniinae - Rhiniinae	-0.569	0.57	0.747
		Bengaliinae - Rhiniinae	0.95	0.342	0.599
		Calliphorinae - Rhiniinae	-1.209	0.227	0.433
		Chrysomyinae - Rhiniinae	-0.307	0.759	0.839
		Luciliinae - Rhiniinae	0.232	0.817	0.817
		Ameniinae - Rhinophorinae	1.856	0.063	0.19
		Bengaliinae - Rhinophorinae	3.542	0	**0.008**
		Calliphorinae - Rhinophorinae	2.153	0.031	0.131
		Chrysomyinae - Rhinophorinae	2.118	0.034	0.12
		Luciliinae - Rhinophorinae	2.657	0.008	**0.055**
		Rhiniinae - Rhinophorinae	2.425	0.015	0.08
	GC-skew	Ameniinae - Bengaliinae	1.182	0.237	0.383
		Ameniinae - Calliphorinae	-0.869	0.385	0.505
		Bengaliinae - Calliphorinae	-2.757	0.006	**0.041**
		Ameniinae - Chrysomyinae	-0.097	0.923	0.969
		Bengaliinae - Chrysomyinae	-1.286	0.198	0.379
		Calliphorinae - Chrysomyinae	0.734	0.463	0.572
		Ameniinae - Luciliinae	1.287	0.198	0.416
		Bengaliinae - Luciliinae	0.194	0.846	0.987
		Calliphorinae - Luciliinae	2.654	0.008	**0.042**
		Chrysomyinae - Luciliinae	1.384	0.166	0.388
		Ameniinae - Rhiniinae	0.045	0.964	0.964
		Bengaliinae - Rhiniinae	-1.134	0.257	0.385
		Calliphorinae - Rhiniinae	0.931	0.352	0.492
		Chrysomyinae - Rhiniinae	0.142	0.887	0.98
		Luciliinae - Rhiniinae	-1.242	0.214	0.375
		Ameniinae - Rhinophorinae	-1.811	0.07	0.246
		Bengaliinae - Rhinophorinae	-3.118	0.002	**0.038**
		Calliphorinae - Rhinophorinae	-1.642	0.101	0.264
		Chrysomyinae - Rhinophorinae	-1.714	0.087	0.26
		Luciliinae - Rhinophorinae	-3.098	0.002	**0.02**
		Rhiniinae - Rhinophorinae	-1.856	0.063	0.267
Protein-coding genes	**A+T %**	Ameniinae - Bengaliinae	1.32	0.187	0.302
		Ameniinae - Calliphorinae	2.045	0.041	0.143
		Bengaliinae - Calliphorinae	0.377	0.706	0.824
		Ameniinae - Chrysomyinae	1.394	0.163	0.312
		Bengaliinae - Chrysomyinae	0.17	0.865	0.908
		Calliphorinae - Chrysomyinae	-0.108	0.914	0.914
		Ameniinae - Luciliinae	2.46	0.014	0.073
		Bengaliinae - Luciliinae	1.309	0.19	0.286
		Calliphorinae - Luciliinae	1.374	0.169	0.297
		Chrysomyinae - Luciliinae	1.066	0.286	0.401
		Ameniinae - Rhiniinae	-0.273	0.785	0.867
		Bengaliinae - Rhiniinae	-1.612	0.107	0.224
		Calliphorinae - Rhiniinae	-2.425	0.015	0.064

		Chrysomyinae - Rhiniinae	-1.667	0.095	0.223
		Luciliinae - Rhiniinae	-2.733	0.006	0.066
		Ameniinae - Rhinophorinae	-0.623	0.533	0.7
		Bengaliinae - Rhinophorinae	-1.821	0.069	0.18
		Calliphorinae - Rhinophorinae	-2.475	0.013	0.093
		Chrysomyinae - Rhinophorinae	-1.87	0.061	0.184
		Luciliinae - Rhinophorinae	-2.823	0.005	0.1
		Rhiniinae - Rhinophorinae	-0.379	0.705	0.871
	G+C %	Ameniinae - Bengaliinae	-1.32	0.187	0.302
		Ameniinae - Calliphorinae	-2.045	0.041	0.143
		Bengaliinae - Calliphorinae	-0.377	0.706	0.824
		Ameniinae - Chrysomyinae	-1.394	0.163	0.312
		Bengaliinae - Chrysomyinae	-0.17	0.865	0.908
		Calliphorinae - Chrysomyinae	0.108	0.914	0.914
		Ameniinae - Luciliinae	-2.46	0.014	0.073
		Bengaliinae - Luciliinae	-1.309	0.19	0.286
		Calliphorinae - Luciliinae	-1.374	0.169	0.297
		Chrysomyinae - Luciliinae	-1.066	0.286	0.401
		Ameniinae - Rhiniinae	0.273	0.785	0.867
		Bengaliinae - Rhiniinae	1.612	0.107	0.224
		Calliphorinae - Rhiniinae	2.425	0.015	0.064
		Chrysomyinae - Rhiniinae	1.667	0.095	0.223
		Luciliinae - Rhiniinae	2.733	0.006	0.066
		Ameniinae - Rhinophorinae	0.623	0.533	0.7
		Bengaliinae - Rhinophorinae	1.821	0.069	0.18
		Calliphorinae - Rhinophorinae	2.475	0.013	0.093
		Chrysomyinae - Rhinophorinae	1.87	0.061	0.184
		Luciliinae - Rhinophorinae	2.823	0.005	0.1
		Rhiniinae - Rhinophorinae	0.379	0.705	0.871
	AT-skew	Ameniinae - Bengaliinae	1.101	0.271	0.379
		Ameniinae - Calliphorinae	0.446	0.655	0.688
		Bengaliinae - Calliphorinae	-1.131	0.258	0.452
		Ameniinae - Chrysomyinae	1.131	0.258	0.417
		Bengaliinae - Chrysomyinae	0.108	0.914	0.914
		Calliphorinae - Chrysomyinae	1.125	0.26	0.391
		Ameniinae - Luciliinae	1.624	0.104	0.244
		Bengaliinae - Luciliinae	0.635	0.525	0.649
		Calliphorinae - Luciliinae	1.811	0.07	0.245
		Chrysomyinae - Luciliinae	0.493	0.622	0.687
		Ameniinae - Rhiniinae	-0.541	0.588	0.686
		Bengaliinae - Rhiniinae	-1.679	0.093	0.279
		Calliphorinae - Rhiniinae	-1.199	0.231	0.44
		Chrysomyinae - Rhiniinae	-1.672	0.095	0.248
		Luciliinae - Rhiniinae	-2.165	0.03	0.128
		Ameniinae - Rhinophorinae	-1.548	0.122	0.256
		Bengaliinae - Rhinophorinae	-2.602	0.009	0.097
		Calliphorinae - Rhinophorinae	-2.341	0.019	0.101
		Chrysomyinae - Rhinophorinae	-2.559	0.011	0.074
		Luciliinae - Rhinophorinae	-3	0.003	0.057
		Rhiniinae - Rhinophorinae	-1.063	0.288	0.377
	GC-skew	Ameniinae - Bengaliinae	-1.177	0.239	0.502
		Ameniinae - Calliphorinae	-1.763	0.078	0.272
		Bengaliinae - Calliphorinae	-0.268	0.789	0.828

		Ameniinae - Chrysomyinae	-1.634	0.102	0.307
		Bengaliinae - Chrysomyinae	-0.57	0.569	0.746
		Calliphorinae - Chrysomyinae	-0.508	0.612	0.755
		Ameniinae - Luciliinae	-0.451	0.652	0.76
		Bengaliinae - Luciliinae	0.694	0.487	0.731
		Calliphorinae - Luciliinae	1.136	0.256	0.488
		Chrysomyinae - Luciliinae	1.183	0.237	0.553
		Ameniinae - Rhiniinae	-2.947	0.003	0.067
		Bengaliinae - Rhiniinae	-1.974	0.048	0.203
		Calliphorinae - Rhiniinae	-2.332	0.02	0.138
		Chrysomyinae - Rhiniinae	-1.313	0.189	0.497
		Luciliinae - Rhiniinae	-2.496	0.013	0.132
		Ameniinae - Rhinophorinae	-0.37	0.711	0.786
		Bengaliinae - Rhinophorinae	0.648	0.517	0.724
		Calliphorinae - Rhinophorinae	0.976	0.329	0.532
		Chrysomyinae - Rhinophorinae	1.092	0.275	0.481
		Luciliinae - Rhinophorinae	0.034	0.973	0.973
		Rhiniinae - Rhinophorinae	2.266	0.023	0.123

Comparison: Labels for each pairwise comparison.

Z: Values for the Z test statistic for each comparison.

P.unadj: Unadjusted p-values for each comparison.

P.adj: Adjusted p-values for each comparison.

Table S7. Most frequently used codons (RSCU>1) and corresponding tRNA anticodons in Calliphoridae mitogenomes. For improved readability, the table has been divided into two parts, Table S7(A), consisting data for 11 tRNAs (Phe, Leu2, Ile, Met, Val, Ser2, Pro, Thr, Ala, Tyr and His) and Table S7(B), consisting data for the remaining 10 tRNAs (Gln, Asn, Lys, Asp, Glu, Cys, Trp, Arg, Ser1, Gly).

Table S7(A)

ID	Subfamily	Species	GenBank Accession	Most frequently used codons (RSCU values) and anticodons on corresponding tRNAs										
				Phe: UUU (1.74)	Leu2: UUA (4.83)	Ile: AUU (1.87)	Met: AUA (1.77)	Val: GUU/ GUA (1.74/ 2.03)	Ser2: UCU/ UCA (2.56/ 2.28)	Pro: CCU/ CCA (2.17 /1.52)	Thr: ACU/ ACA (1.99/ 1.71)	Ala: GCU/ GCA (2.49/ 1.15)	Tyr: UAU (1.7)	His: CAU (1.64)
KEIB_DIP_02070	Calliphorinae	*Calliphora* sp. n. 3	PQ593971	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02099	Calliphorinae	*Calliphora alaskensis*	PQ593972	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02102	Calliphorinae	*Calliphora calliphoroides*	PQ593973	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02104	Calliphorinae	*Calliphora coloradensis*	PQ593974	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02109	Calliphorinae	*Calliphora genarum*	PQ593975	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02110	Calliphorinae	*Calliphora grahami*	PQ593976	gaa	uaa	gau	cau	uac	uga	ugg	ugu	tgc	gua	gug
KEIB_DIP_02112	Calliphorinae	*Calliphora hilli*	PQ593977	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02113	Calliphorinae	*Calliphora himalayana*	PQ593978	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02122	Calliphorinae	*Calliphora calliphoroides*	PQ594007	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug

KEIB_DIP_02124	Calliphorinae	*Calliphora nigrithorax*	PQ593979	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02130	Calliphorinae	*Calliphora rohdendorfi*	PQ593980	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02135	Calliphorinae	*Calliphora subalpina*	PQ593981	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02136	Calliphorinae	*Calliphora subalpina*	PQ593982	gaa	uaa	gat **(duplicated)**	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02138	Calliphorinae	*Calliphora terraenovae*	PQ594006	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02142	Calliphorinae	*Calliphora vicina*	PQ593983	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02143	Calliphorinae	*Calliphora varifrons*	PQ594010	gaa	taa	gat	cat	tac	tga	tgg	tgt	tgc	gta	gtg
KEIB_DIP_02146	Calliphorinae	*Calliphora teraramma*	PQ593984	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02147	Calliphorinae	*Calliphora mesay*	PQ593985	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02148	Calliphorinae	*Calliphora mesay*	PQ593986	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02150	Calliphorinae	*Calliphora teraramma*	PQ593987	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02151	Calliphorinae	*Calliphora teraramma*	PQ593988	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_01527	Calliphorinae	*Calliphora vicina*	PQ593989	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_01540	Calliphorinae	*Calliphora vomitoria*	PQ593990	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_01711	Calliphorinae	*Calliphora latifrons*	PQ593991	gaa	uaa	gau **(duplicated)**	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_01712	Calliphorinae	*Calliphora irazuana*	PQ593992	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug

KEIB_DIP_01672	Calliphorinae	*Calliphora uralensis*	PQ593993	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_01571	Calliphorinae	*Calliphora loewi*	PQ594008	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02015	Calliphorinae	*Calliphora albifrontalis*	PQ593994	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02018	Calliphorinae	*Calliphora rohdendorfi*	PQ593995	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02026	Calliphorinae	*Calliphora ochracea*	PQ593996	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02027	Calliphorinae	*Calliphora dubia*	PQ593997	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02029	Calliphorinae	*Calliphora quadrimaculata*	PQ593998	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02030	Calliphorinae	*Xenocalliphora neozelandica*	PQ593999	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02032	Calliphorinae	*Calliphora hilli*	PQ594000	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02033	Calliphorinae	*Calliphora sternalis*	PQ594001	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_01463	Calliphorinae	*Calliphora subalpina*	PQ594009	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02043	Calliphorinae	*Calliphora croceipalpis*	PQ594002	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02085	Calliphorinae	*Onesia zumpti*	PQ594003	gaa **(duplicated)**	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_01557	Calliphorinae	*Onesia floralis*	PQ635404	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_01619	Calliphorinae	*Onesia accepta*	PQ635405	gaa **(duplicated)**	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02016	Calliphorinae	*Melinda viridicyanea*	PQ635406	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02036	Calliphorinae	*Onesia tibialis*	PQ635407	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02037	Calliphorinae	*Onesia minor*	PQ635408	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug

KEIB_DIP_02038	Calliphorinae	*Onesia austriaca*	PQ635409	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02039	Calliphorinae	*Onesia floralis*	PQ635410	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02091	Calliphorinae	*Bellardia bayeri*	PQ635411	gaa	uaa	gau **(partially duplicated)**	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02093	Calliphorinae	*Bellardia pubicornis*	PQ635412	gaa	taa	gat	cat	tac	tga	tgg	tgt	tgc	gta	gtg
KEIB_DIP_02095	Calliphorinae	*Bellardia siciliensis*	PQ635413	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_01528	Calliphorinae	*Bellardia vulgaris*	PQ664927	gaa	taa	gat	cat	tac	tga	tgg	tgt	tgc	gta	gtg
KEIB_DIP_02013	Calliphorinae	*Bellardia viarum*	PQ664928	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02014	Calliphorinae	*Bellardia pandia*	PQ664929	gaa	uaa	**Loss**	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02072	Calliphorinae	*Cyanus elongata*	PQ664930	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02074	Calliphorinae	*Cynomya cadaverina*	PQ664931	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02028	Calliphorinae	*Cynomya mortuorum*	PQ664986	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_01969	Calliphorinae	*Pericallimyia* sp.	PQ665286	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02020	Calliphorinae	*Melinda viridicyanea*	PQ664953	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02023	Calliphorinae	*Paradichosia okazaki*	PQ664954	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_01822	Calliphorinae	*Eggisops pecchiolii*	PQ664955	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02041	Calliphorinae	*Sarconesia magellanica*	PQ649436	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug

KEIB_DIP_01987	Luciliinae	*Hemipyrellia fernandica*	PQ649437	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_01636	Luciliinae	*Lucilia porphyrina*	PQ649438	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02007	Luciliinae	*Lucilia eximia*	PQ649439	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02268	Chrysomyinae	*Cochliomyia minima*	PQ649440	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02272	Chrysomyinae	*Compsomyiops fulvicrura*	PQ664987	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_01559	Chrysomyinae	*Chrysomya albiceps*	PQ665287	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02009	Ameniinae	*Amenia imperialis*	PQ665281	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02012	Ameniinae	*Paramenia semiauriceps*	PQ665282	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_02017	Ameniinae	*Eurychaeta palpalis*	PQ665283	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_01983	Bengaliinae	*Tricyclea* sp. 2	PQ654328	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_01980	Bengaliinae	*Cordylobia anthropophaga*	PQ654329	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_01981	Bengaliinae	*Bengalia seniorwhitei*	PQ654330	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_01707	Phumosinae	*Phumosia promittens*	PQ654331	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_01692	Rhiniinae	*Rhyncomya zumpti*	PQ654332	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KP872701	Calliphorinae	*Calliphora grahami*	KP872701	gaa	taa	gat	cat	tac	tga	tgg	tgt	ugc	gta	gtg
OU696535	Calliphorinae	*Bellardia pandia*	OU696535	gaa	taa	gat	cat **(duplicated)**	tac	tga	tgg	tgt	tgc	gta	gtg

OX493271	Calliphorinae	*Bellardia bayeri*	OX493271	gaa	taa	gat **(3 copies)**	cat **(partially duplicated)**	tac	tga	tgg	tgt	tgc	gta	gtg
OY288238	Calliphorinae	*Calliphora vicina*	OY288238	gaa	taa	gat	cat	tac	tga	tgg	tgt	tgc	gta	gtg
MT017724	Calliphorinae	*Calliphora nigribarbis*	MT017724	gaa	taa	gat	cat	tac	tga	tgg	tgt	tgc	gta	gtg
MT017707	Calliphorinae	*Calliphora sinensis*	MT017707	gaa	taa	gat	cat	tac	tga	tgg	tgt	tgc	gta	gtg
MT017722	Calliphorinae	*Calliphora uralensis*	MT017722	gaa	taa	gat	cat	tac	tga	tgg	tgt	tgc	gta	gtg
MT628574	Calliphorinae	*Cynomya mortuorum*	MT628574	gaa	taa	gat	cat	tac	tga	tgg	tgt	tgc	gta	gtg
MN131052	Calliphorinae	*Polleniopsis mongolica*	MN131052	gaa	taa	gat	cat	tac	tga	tgg	tgt	tgc	gta	gtg
MK893471	Calliphorinae	*Calliphora calliphoroides*	MK893471	gaa	taa	gat **(duplicated)**	cat	tac	tga	tgg	tgt	tgc	gta	gtg
MK591038	Bengalinae	*Bengalia* sp.	MK591038	gaa	taa	gat	cat	tac	tga	tgg	tgt	tgc	gta	gtg
OW121746	Rhiniinae	*Stomorhina lunata*	OW121746	gaa	taa	gat	cat	tac	tga	tgg	tgt	tgc	gta	gtg
OR497843	Rhiniinae	*Isomyia nebulosa*	OR497843	gaa	taa	gat	cat	tac	tga	tgg	tgt	tgc	gta	gtg
OW799239	Rhinophorinae	*Phyto melanocephala*	OW799239	gaa	taa	gat	cat	tac	tga	tgg	tgt	tgc	gta	gtg
OY757193	Rhinophorinae	*Melanophora roralis*	OY757193	gaa	taa	gat	cat	tac	tga	tgg	tgt	tgc	gta	gtg
KEIB_DIP_02118	Calliphorinae	*Calliphora loewi*	PQ654167	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_01594	Calliphorinae	*Bellardia stricta*	PQ654168	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug
KEIB_DIP_01541	Rhinophorinae	*Rhinomorinia sarcophagina*	PQ654169	gaa	uaa	gau	cau	uac	uga	ugg	ugu	ugc	gua	gug

MT584151	Calliphorinae	*Calliphora vomitoria*	MT584151	gaa	taa	gat	cat	tac	tga	tgg	tgt	tgc	gta	gtg
KT936147	Calliphorinae	*Calliphora chinghaiensis*	KT936147	gaa	taa	gat	cat	tac	tga	tgg	tgt	tgc	gta	gtg

Table S7(B)

ID	**Subfamily**	**Species**	**GenBank Accession**	**Most frequently used codons (RSCU values) and anticodons on corresponding tRNAs**									
				Gln: CAA (1.87)	**Asn: AAU (1.76)**	**Lys: AAA (1.51)**	**Asp: GAU (1.63)**	**Glu: GAA (1.89)**	**Cys: UGU (1.73)**	**Trp: UGA (1.78)**	**Arg: CGA (2.95)**	**Ser1: AGU/ AGA (1.16/ 1.24)**	**Gly: GGA (2.51)**
KEIB_DIP_02070	Calliphorinae	*Calliphora* sp. n. 3	PQ593971	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02099	Calliphorinae	*Calliphora alaskensis*	PQ593972	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02102	Calliphorinae	*Calliphora calliphoroides*	PQ593973	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02104	Calliphorinae	*Calliphora coloradensis*	PQ593974	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02109	Calliphorinae	*Calliphora genarum*	PQ593975	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02110	Calliphorinae	*Calliphora grahami*	PQ593976	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02112	Calliphorinae	*Calliphora hilli*	PQ593977	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02113	Calliphorinae	*Calliphora himalayana*	PQ593978	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02122	Calliphorinae	*Calliphora calliphoroides*	PQ594007	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02124	Calliphorinae	*Calliphora nigrithorax*	PQ593979	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02130	Calliphorinae	*Calliphora rohdendorfi*	PQ593980	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02135	Calliphorinae	*Calliphora subalpina*	PQ593981	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02136	Calliphorinae	*Calliphora subalpina*	PQ593982	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02138	Calliphorinae	*Calliphora terraenovae*	PQ594006	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02142	Calliphorinae	*Calliphora vicina*	PQ593983	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02143	Calliphorinae	*Calliphora varifrons*	PQ594010	ttg	gtt	ctt	gtc	ttc	gca	tca	tcg	gct	tcc

KEIB_DIP_02146	Calliphorinae	*Calliphora teraramma*	PQ593984	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02147	Calliphorinae	*Calliphora mesay*	PQ593985	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02148	Calliphorinae	*Calliphora mesay*	PQ593986	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02150	Calliphorinae	*Calliphora teraramma*	PQ593987	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02151	Calliphorinae	*Calliphora teraramma*	PQ593988	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_01527	Calliphorinae	*Calliphora vicina*	PQ593989	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_01540	Calliphorinae	*Calliphora vomitoria*	PQ593990	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_01711	Calliphorinae	*Calliphora latifrons*	PQ593991	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_01712	Calliphorinae	*Calliphora irazuana*	PQ593992	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_01672	Calliphorinae	*Calliphora uralensis*	PQ593993	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_01571	Calliphorinae	*Calliphora loewi*	PQ594008	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02015	Calliphorinae	*Calliphora albifrontalis*	PQ593994	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02018	Calliphorinae	*Calliphora rohdendorfi*	PQ593995	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02026	Calliphorinae	*Calliphora ochracea*	PQ593996	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02027	Calliphorinae	*Calliphora dubia*	PQ593997	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02029	Calliphorinae	*Calliphora quadrimaculata*	PQ593998	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02030	Calliphorinae	*Xenocalliphora neozelandica*	PQ593999	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02032	Calliphorinae	*Calliphora hilli*	PQ594000	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02033	Calliphorinae	*Calliphora sternalis*	PQ594001	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_01463	Calliphorinae	*Calliphora subalpina*	PQ594009	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02043	Calliphorinae	*Calliphora croceipalpis*	PQ594002	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02085	Calliphorinae	*Onesia zumpti*	PQ594003	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_01557	Calliphorinae	*Onesia floralis*	PQ635404	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_01619	Calliphorinae	*Onesia accepta*	PQ635405	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02016	Calliphorinae	*Melinda viridicyanea*	PQ635406	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02036	Calliphorinae	*Onesia tibialis*	PQ635407	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02037	Calliphorinae	*Onesia minor*	PQ635408	ttg	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02038	Calliphorinae	*Onesia austriaca*	PQ635409	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02039	Calliphorinae	*Onesia floralis*	PQ635410	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02091	Calliphorinae	*Bellardia bayeri*	PQ635411	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc

KEIB_DIP_02093	Calliphorinae	*Bellardia pubicornis*	PQ635412	ttg	gtt	ctt	gtc	ttc	gca	tca	tcg	gct	tcc
KEIB_DIP_02095	Calliphorinae	*Bellardia siciliensis*	PQ635413	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_01528	Calliphorinae	*Bellardia vulgaris*	PQ664927	ttg	gtt	ctt	gtc	ttc	gca	tca	tcg	gct	tcc
KEIB_DIP_02013	Calliphorinae	*Bellardia viarum*	PQ664928	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02014	Calliphorinae	*Bellardia pandia*	PQ664929	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02072	Calliphorinae	*Cyanus elongata*	PQ664930	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02074	Calliphorinae	*Cynomya cadaverina*	PQ664931	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02028	Calliphorinae	*Cynomya mortuorum*	PQ664986	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_01969	Calliphorinae	*Pericallimyia* sp.	PQ665286	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02020	Calliphorinae	*Melinda viridicyanea*	PQ664953	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_02023	Calliphorinae	*Paradichosia okazaki*	PQ664954	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
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KEIB_DIP_02041	Calliphorinae	*Sarconesia magellanica*	PQ649436	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_01987	Luciliinae	*Hemipyrellia fernandica*	PQ649437	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
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KEIB_DIP_02272	Chrysomyinae	*Compsomyiops fulvicrura*	PQ664987	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_01559	Chrysomyinae	*Chrysomya albiceps*	PQ665287	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
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KEIB_DIP_01707	Phumosinae	*Phumosia promittens*	PQ654331	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
KEIB_DIP_01692	Rhiniinae	*Rhyncomya zumpti*	PQ654332	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
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OU696535	Calliphorinae	*Bellardia pandia*	OU696535	ttg **(duplicated)**	gtt	ctt	gtc	ttc	gca	tca	tcg	gct	tcc

OX493271	Calliphorinae	*Bellardia bayeri*	OX493271	ttg **(duplicated)**	gtt	ctt	gtc	ttc	gca	tca	tcg	gct	tcc
OY288238	Calliphorinae	*Calliphora vicina*	OY288238	ttg	gtt	ctt	gtc	ttc	gca	tca	tcg	gct	tcc
MT017724	Calliphorinae	*Calliphora nigribarbis*	MT017724	ttg	gtt	ctt	gtc	ttc	gca	tca	tcg	gct	tcc
MT017707	Calliphorinae	*Calliphora sinensis*	MT017707	ttg	gtt	ctt	gtc	ttc	gca	tca	tcg	gct	tcc
MT017722	Calliphorinae	*Calliphora uralensis*	MT017722	ttg	gtt	ctt	gtc	ttc	gca	tca	tcg	gct	tcc
MT628574	Calliphorinae	*Cynomya mortuorum*	MT628574	ttg	gtt	ctt	gtc	ttc	gca	tca	tcg	gct	tcc
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MK591038	Bengalinae	*Bengalia* sp.	MK591038	ttg	gtt	ctt	gtc	ttc	gca	tca	tcg	gct	tcc
OW121746	Rhiniinae	*Stomorhina lunata*	OW121746	ttg	gtt	ctt	gtc	ttc	gca	tca	tcg	gct	tcc
OR497843	Rhiniinae	*Isomyia nebulosa*	OR497843	ttg	gtt	ctt	gtc	ttc	gca	tca	tcg	gct	tcc
OW799239	Rhinophorinae	*Phyto melanocephala*	OW799239	ttg	gtt	ctt	gtc	ttc	gca	tca	tcg	gct	tcc
OY757193	Rhinophorinae	*Melanophora roralis*	OY757193	ttg	gtt	ctt	gtc	ttc	gca	tca	tcg	gct	tcc
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KEIB_DIP_01541	Rhinophorinae	*Rhinomorinia sarcophagina*	PQ654169	uug	guu	cuu	guc	uuc	gca	uca	ucg	gcu	ucc
MT584151	Calliphorinae	*Calliphora vomitoria*	MT584151	ttg	gtt	ctt	gtc	ttc	gca	tca	tcg	gct	tcc
KT936147	Calliphorinae	*Calliphora chinghaiensis*	KT936147	ttg	gtt	ctt	gtc	ttc	gca	tca	tcg	gct	tcc

Table S8. Approximately unbiased (AU) topology tests for 10,000 replicates under the model estimated by IQ-TREE.

Tree	logL	bp-RELL	deltaL	p-KH	p-SH	p-AU
1 (Unconstrained - Polyphyletic *Calliphora*)	-176400.3556	1 +	0	1 +	1 +	1 +
2 (Constrained - Monophyletic *Calliphora*)	-176569.0489	0 -	168.69	0 -	0 -	1.81e-37 -

deltaL: logL difference from the maximal logl in the set

bp-RELL: bootstrap proportion using RELL method (Kishino et al. 1990).

p-KH: p-value of one sided Kishino-Hasegawa test (1989).

p-SH: p-value of Shimodaira-Hasegawa test (2000).

p-AU: p-value of approximately unbiased (AU) test (Shimodaira, 2002).

Plus (+) signs denote the 95% confidence sets.

Minus (-) signs denote significant exclusion.

All tests performed 10000 resampling using the RELL method.

Supplementary Figures

Fig. S1. Geographic location of the collection sites of specimens used in this study. Green circles represent Calliphorinae specimens, red circles represent specimens from other seven calliphorid subfamilies and blue circles represent specimens used for outgroup in our phylogenetic analysis.

Fig. S2. Relative synonymous codon usage (RSCU) of the mitochondrial protein coding genes of Calliphorinae only.

Fig. S3. Phylogenetic hypothesis for Calliphoridae based on 13 mitochondrial protein-coding genes (including all codon positions), using a maximum likelihood method in the IQ-TREE under AIC-based model selection (ML123). The dataset includes mitogenomes of 84 species (107 taxa), including Mesembrinellidae and Ulurumyiidae as outgroups. Nodal support is given as SH-alrt support/ IQ-TREE ultrafast bootstrap support. The voucher codes or Genbank accession numbers are included along with the species with more than one specimen, for clarity. Colors of the clades represent the corresponding calliphorid subfamilies as indicated in figure legends.

Fig. S4. Phylogenetic hypothesis for Calliphoridae based on 13 mitochondrial protein-coding genes (excluding the third codon), using a maximum likelihood method in the IQ-TREE under AIC-based model selection (ML12). The dataset includes mitogenomes of 84 species (107 taxa), including Mesembrinellidae and Ulurumyiidae as outgroups. Nodal support is given as SH-alrt support/ IQ-TREE ultrafast bootstrap support. The voucher codes or Genbank accession numbers are included along with the species with more than one specimen, for clarity.

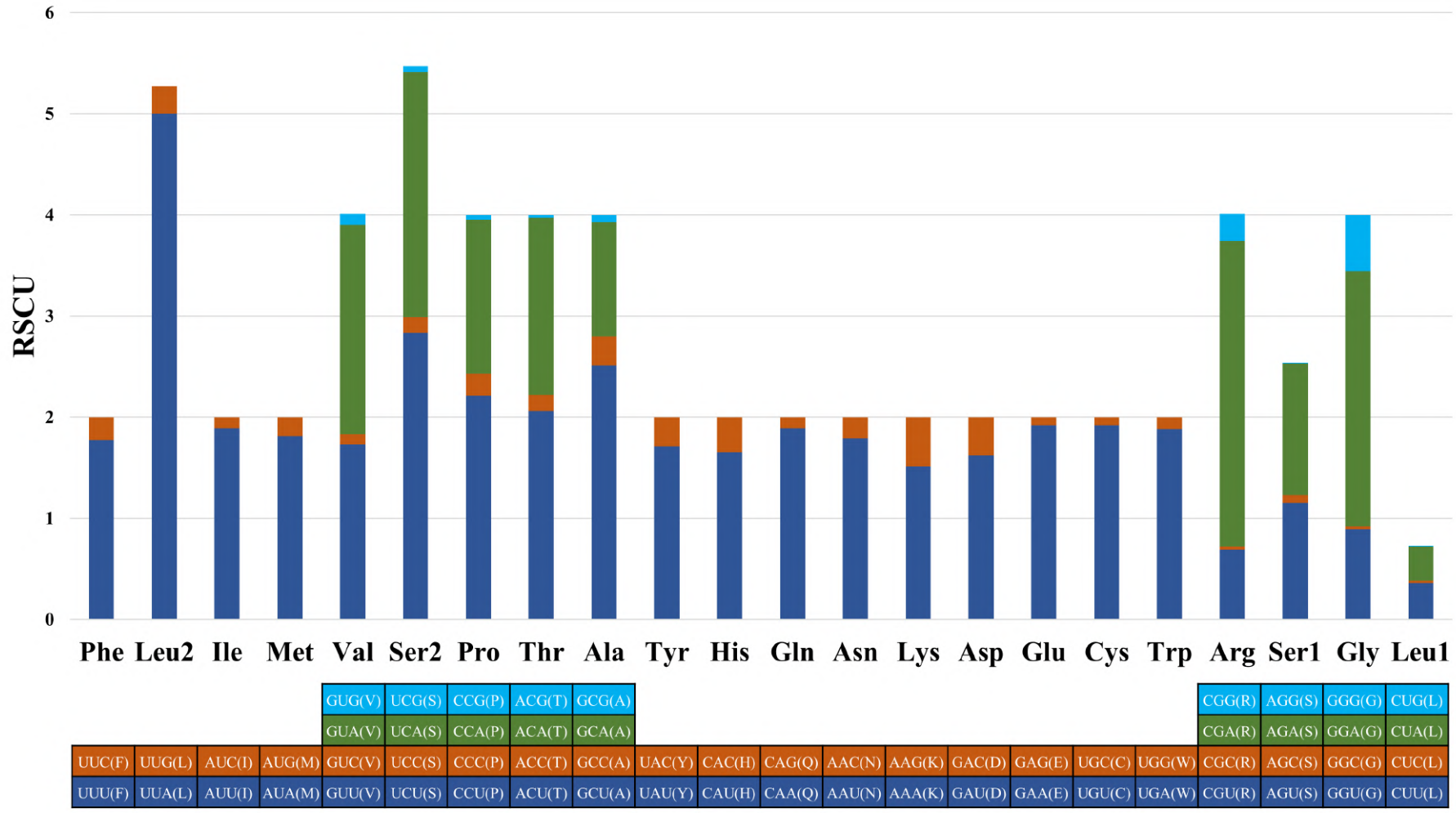
Fig. S5. Phylogenetic hypothesis for Calliphoridae based on 13 mitochondrial protein-coding genes (RY-coded dataset), using a maximum likelihood method in the IQ-TREE under AIC-based model selection (MLRY). The dataset includes mitogenomes of 84 species (107 taxa), including Mesembrinellidae and Ulurumyiidae as outgroups. Nodal support is given as SH-alrt support/ IQ-TREE ultrafast bootstrap support. The voucher codes or Genbank accession numbers are included along with the species with more than one specimen, for clarity.

Fig. S6. Phylogenetic hypothesis for Calliphoridae based on 13 mitochondrial protein-coding genes (excluding the third codon), using a Bayesian inference in ExaBayes (BI12). The dataset includes mitogenomes of 84 species (107 taxa), including Mesembrinellidae and Ulurumyiidae as outgroups. Nodal support is given as ExaBayes posterior probabilities. The voucher codes or Genbank accession numbers are included along with the species with more than one specimen, for clarity.

Fig. S7. Phylogenetic hypothesis for Calliphoridae based on 13 mitochondrial protein-coding genes (RY-coded dataset), using a Bayesian inference in ExaBayes (BIRY). The dataset includes mitogenomes of 84 species (107 taxa), including Mesembrinellidae and Ulurumyiidae as outgroups. Nodal support is given as ExaBayes posterior probabilities. The voucher codes or Genbank accession numbers are included along with the species with more than one specimen, for clarity.


Calliphorinae
Other subfamilies
Outgroup
GREENLAND
ICELAND
CANADA
UNITED STATES
MEXICO
CUBA
GUATEMALA
VENEZUELA
COLOMBIA
GUYANA
ECUADOR
PERU
BRAZIL
BOLIVIA
PARAGUAY
CHILE
URUGUAY
ARGENTINA
Pacific Ocean
Atlantic Ocean
Indian Ocean
NORWAY
SWEDEN
FINLAND
RUSSIA
UNITED KINGDOM
IRELAND
DENMARK
BELARUS
POLAND
BELGIUM
UKRAINE
AUSTRIA
FRANCE
ROMANIA
ITALY
BULGARIA
SPAIN
GREECE
TURKEY
KAZAKHSTAN
MONGOLIA
UZBEKISTAN
TURKMENISTAN
TUNISIA
SYRIA
IRAQ
IRAN
AFGHANISTAN
PAKISTAN
CHINA
NORTH KOREA
SOUTH KOREA
JAPAN
MOROCCO
ALGERIA
LIBYA
EGYPT
ISRAEL
SAUDI ARABIA
UNITED ARAB EMIRATES
NEPAL
BANGLADESH
INDIA
TAIWAN
MAURITANIA
SENEGAL
MALI
NIGER
CHAD
SUDAN
ERITREA
YEMEN
LAOS
MYANMAR
VIETNAM
CAMBODIA
PHILIPPINES
BURKINA FASO
GUINEA
GHANA
NIGERIA
LIBERIA
ETHIOPIA
SOUTH SUDAN
SRI LANKA
MALAYSIA
CAMEROON
KENYA
GABON
RWANDA
INDONESIA
PAPUA NEW GUINEA
TANZANIA
ANGOLA
ZAMBIA
MOZAMBIQUE
NAMIBIA
SOUTH AFRICA
AUSTRALIA
NEW ZEALAND

Fig. S1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GUA(V) UCA(S) CCA(P) ACA(T) GCA(A) CGA(R) AGA(S) GGA(G) CUA(L)
UUC(F) UUG(L) AUC(I) AUG(M) GUC(V) UCC(S) CCC(P) ACC(T) GCC(A) UAC(Y) CAC(H) CAG(Q) AAC(N) AAG(K) GAC(D) GAG(E) UGC(C) UGG(W) CGC(R) AGC(S) GGC(G) CUC(L)
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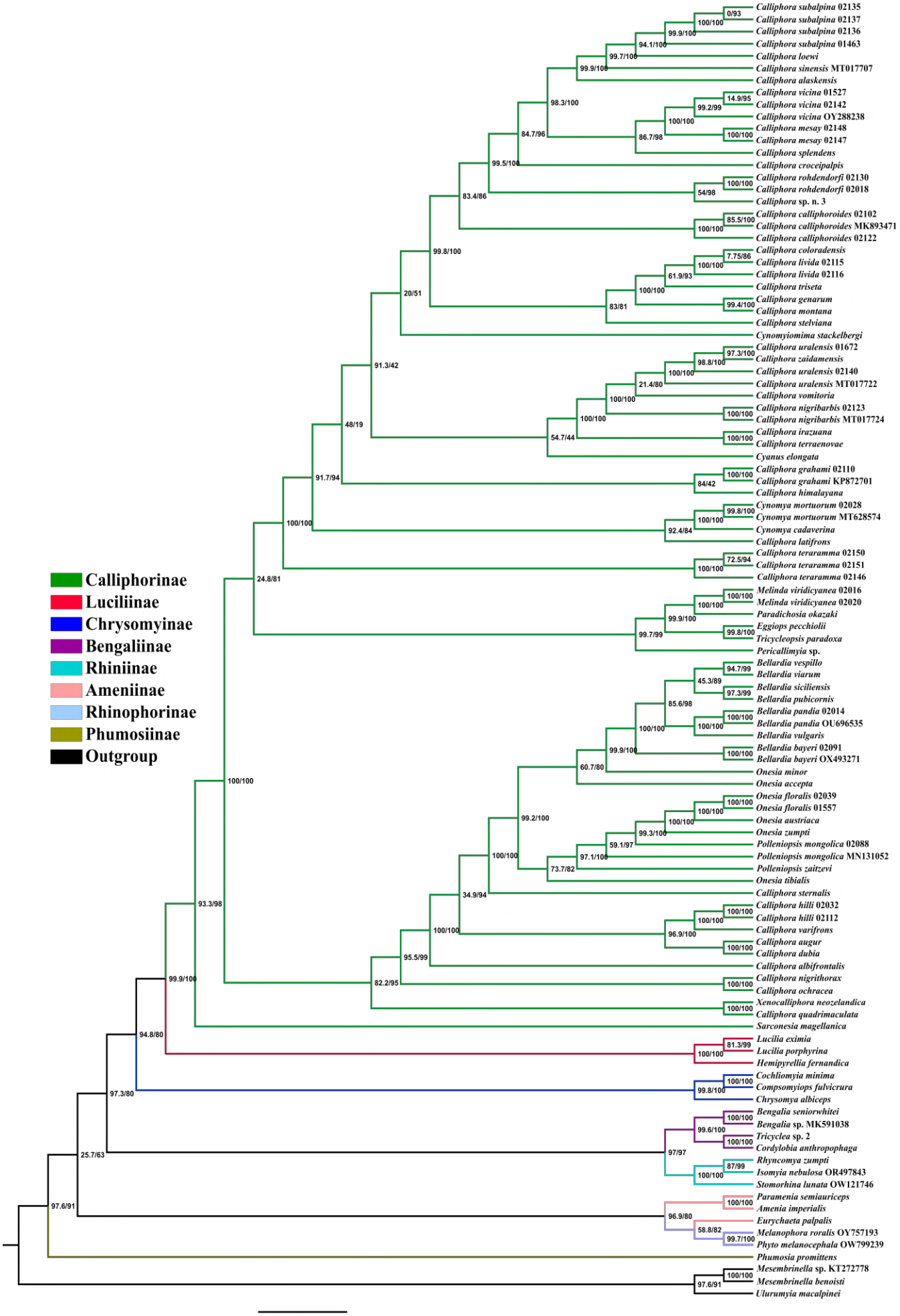
Fig. S2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Bengaliinae
Rhiniinae
Ameniinae
Rhinophorinae
Phumosiinae
Outgroup
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Calliphora subalpina 02136
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Calliphora sinensis MT017707
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Calliphora vomitoria
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Cyanus elongata
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Onesia tibialis
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Calliphora dubia
Calliphora albifrontalis
Calliphora nigrithorax
Calliphora ochracea
Xenocalliphora neozelandica
Calliphora quadrimaculata
Sarconesia magellanica
Lucilia eximia
Lucilia porphyrina
Hemipyrellia fernandica
Cochliomyia minima
Compsomyiops fulvicrura
Chrysomya albiceps
Bengalia seniorwhitei
Bengalia sp. MK591038
Tricyclea sp. 2
Cordylobia anthropophaga
Rhyncomya zumpti
Isomyia nebulosa OR497843
Stomorhina lunata OW121746
Paramenia semiauriceps
Amenia imperialis
Eurychaeta palpalis
Melanophora roralis OY757193
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Fig. S3

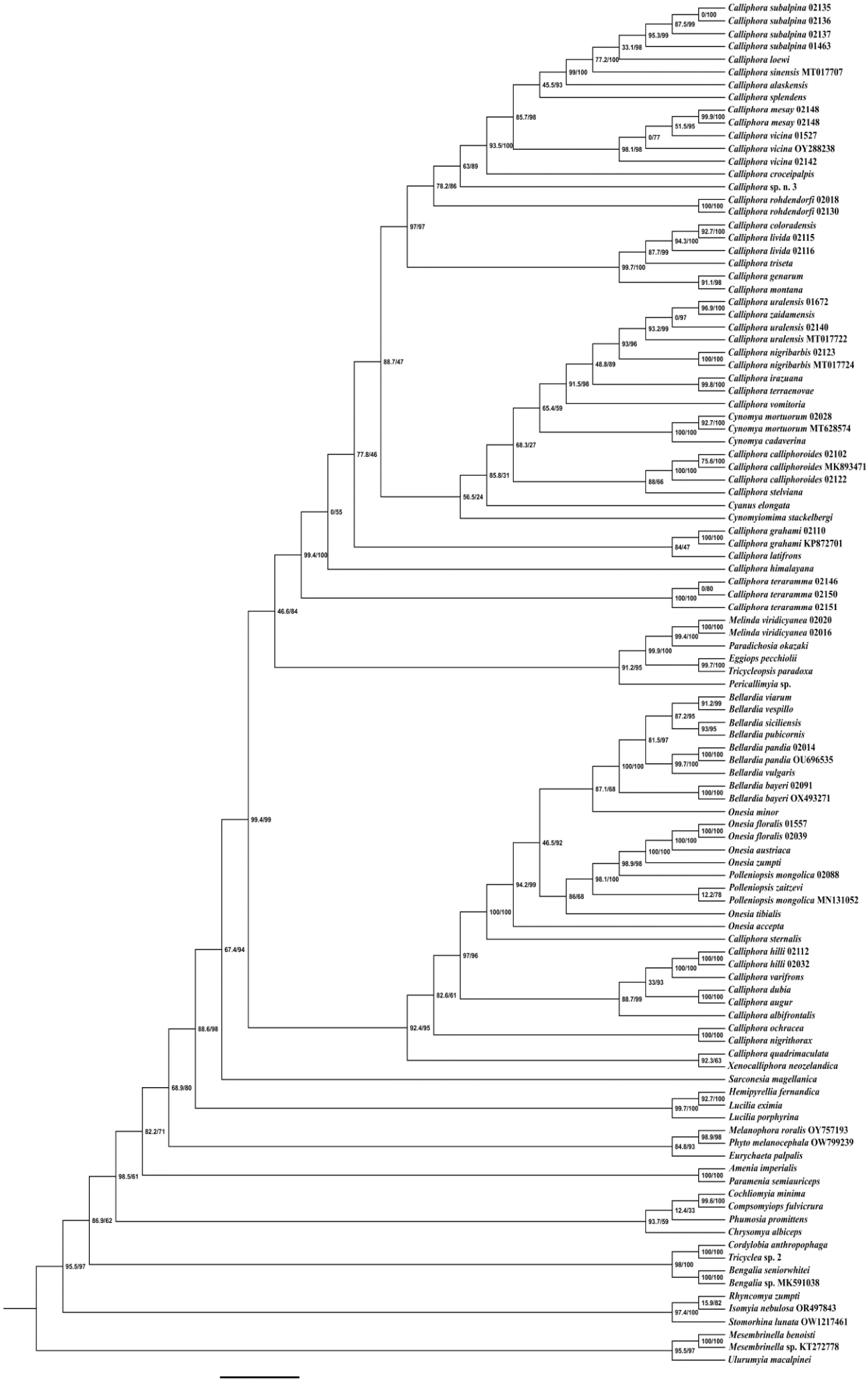
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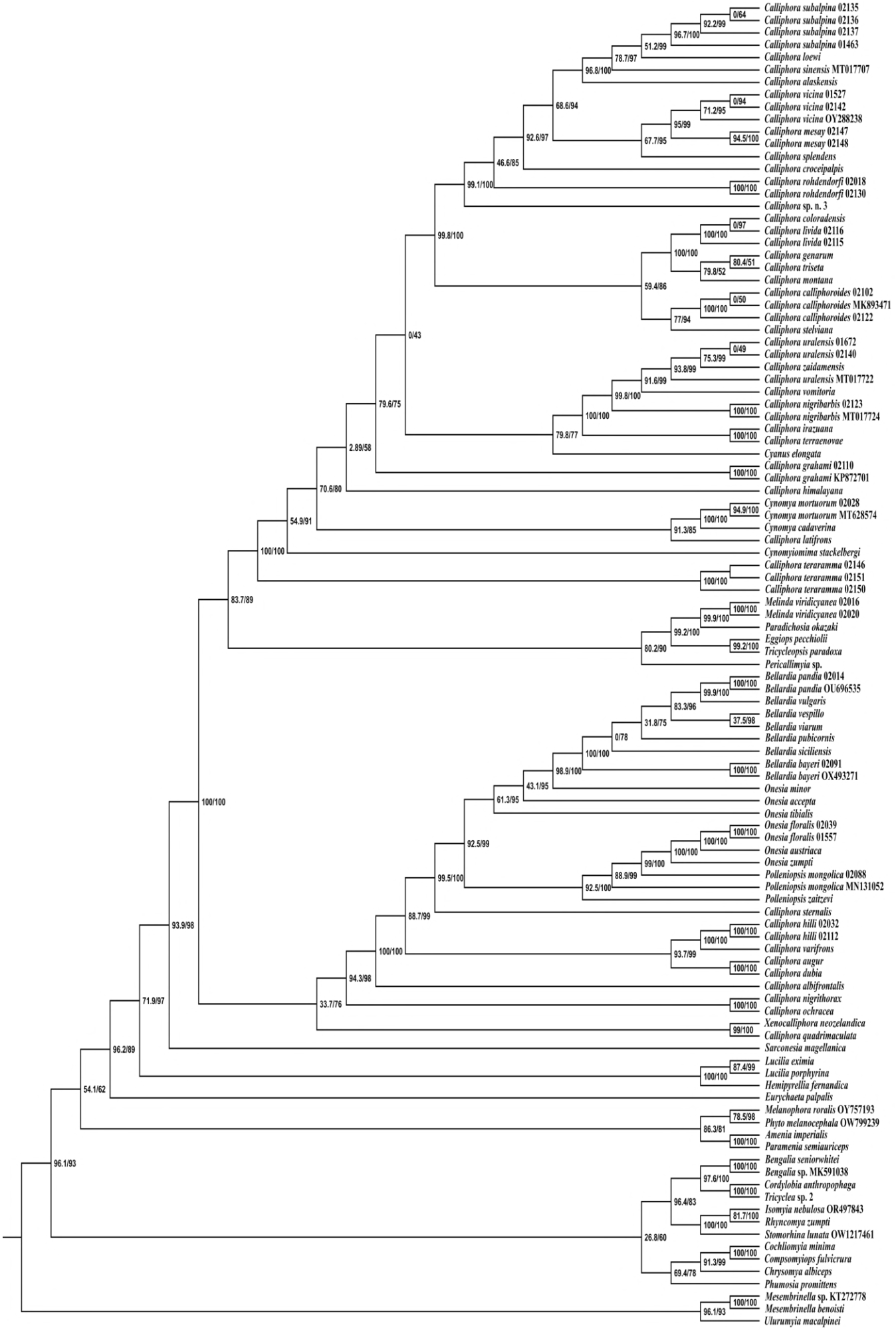
Fig. S4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Calliphora croceipalpis
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Cyanus elongata
Calliphora grahami 02110
Calliphora grahami KP872701
Calliphora himalayana
Cynomya mortuorum 02028
Cynomya mortuorum MT628574
Cynomya cadaverina
Calliphora latifrons
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Calliphora teraramma 02146
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Eggiops pecchiolii
Tricycleopsis paradoxa
Pericallimyia sp.
Bellardia pandia 02014
Bellardia pandia OU696535
Bellardia vulgaris
Bellardia vespillo
Bellardia viarum
Bellardia pubicornis
Bellardia siciliensis
Bellardia bayeri 02091
Bellardia bayeri OX493271
Onesia minor
Onesia accepta
Onesia tibialis
Onesia floralis 02039
Onesia floralis 01557
Onesia austriaca
Onesia zumpti
Polleniopsis mongolica 02088
Polleniopsis mongolica MN131052
Polleniopsis zaitzevi
Calliphora sternalis
Calliphora hilli 02032
Calliphora hilli 02112
Calliphora varifrons
Calliphora augur
Calliphora dubia
Calliphora albifrontalis
Calliphora nigrithorax
Calliphora ochracea
Xenocalliphora neozelandica
Calliphora quadrimaculata
Sarconesia magellanica
Lucilia eximia
Lucilia porphyrina
Hemipyrellia fernandica
Eurychaeta palpalis
Melanophora roralis OY757193
Phyto melanocephala OW799239
Amenia imperialis
Paramenia semiauriceps
Bengalia seniorwhitei
Bengalia sp. MK591038
Cordylobia anthropophaga
Tricyclea sp. 2
Isomyia nebulosa OR497843
Rhyncomya zumpti
Stomorhina lunata OW1217461
Cochliomyia minima
Compsomyiops fulvicrura
Chrysomya albiceps
Phumosia promittens
Mesembrinella sp. KT272778
Mesembrinella benoisti
Ulurumyia macalpinei
0/64
92.2/99
96.7/100
51.2/99
78.7/97
96.8/100
68.6/94
0/94
71.2/95
95/99
94.5/100
67.7/95
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46.6/85
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0/50
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70.6/80
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100/100
99.9/100
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31.8/75
37.5/98
0/78
100/100
98.9/100
100/100
43.1/95
61.3/95
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33.7/76
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99/100
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96.2/89
87.4/99
100/100
71.9/97
54.1/62
78.5/98
86.3/81
100/100
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100/100
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100/100
96.4/83
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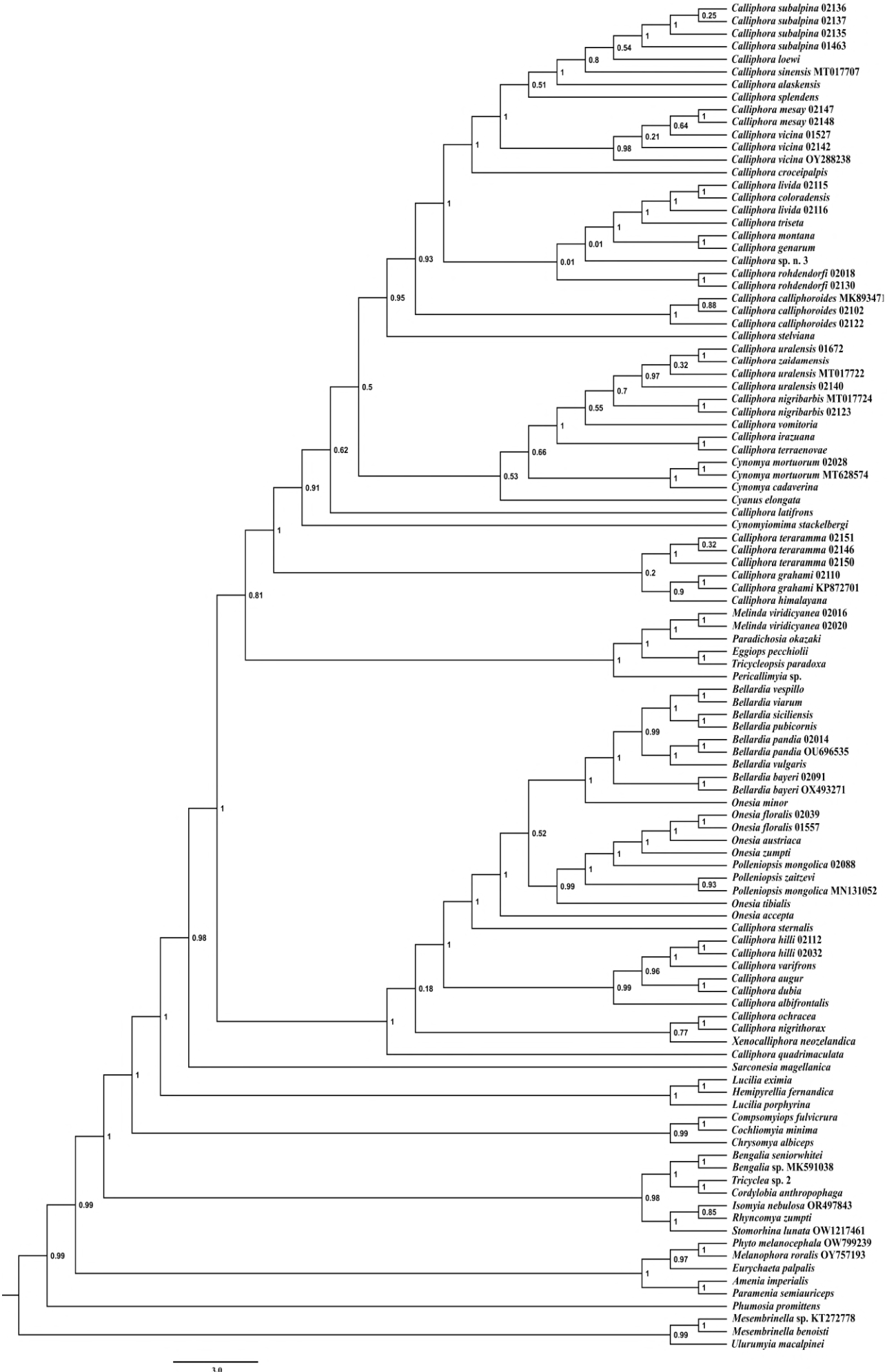
26.8/60
100/100
91.3/99
69.4/78
100/100
96.1/93

130

Fig. S5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Calliphora alaskensis
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Calliphora livida 02115
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Calliphora triseta
Calliphora montana
Calliphora genarum
Calliphora sp. n. 3
Calliphora rohdendorfi 02018
Calliphora rohdendorfi 02130
Calliphora calliphoroides MK893471
Calliphora calliphoroides 02102
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Calliphora stelviana
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Calliphora terraenovae
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Cynomya cadaverina
Cyanus elongata
Calliphora latifrons
Cynomyiomima stackelbergi
Calliphora teraramma 02151
Calliphora teraramma 02146
Calliphora teraramma 02150
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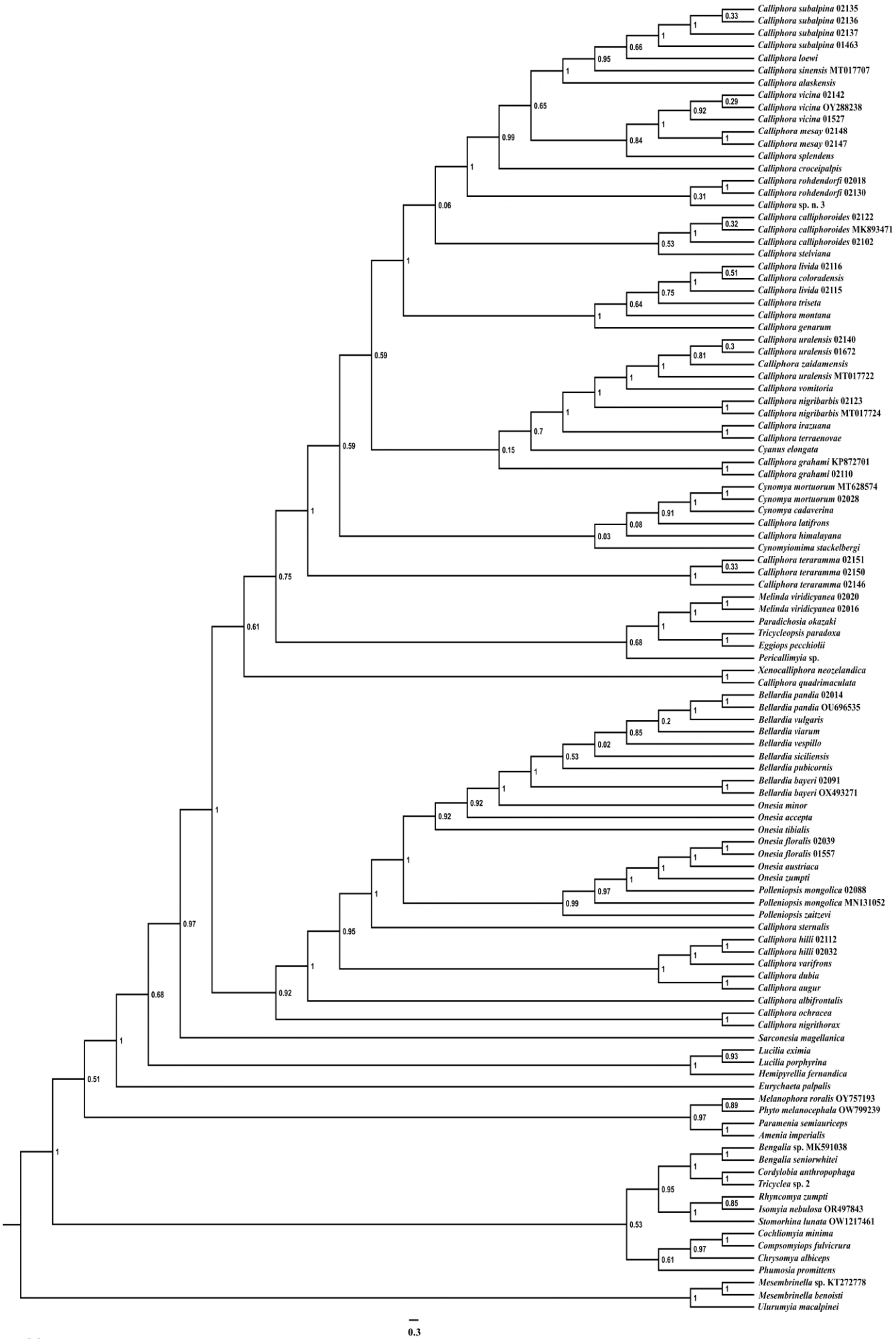
Fig. S6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Mesembrinella benoisti
Ulurumyia macalpinei
0.3

Fig. S7

ARTICLE 3

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ORIGINAL ARTICLE

Medical and Veterinary Entomology

Species delimitation of the Afrotropical and Palaearctic *Calliphora* Robineau-Desvoidy and discovery of two new species in Afrotropics

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Abstract

The blowflies (Calliphoridae) represent a significant portion of schizophoran fly diversity, comprising approximately 2000 known species. Among them, the genus *Calliphora* Robineau-Desvoidy is one of the largest, with over 100 species primarily distributed in the Holarctic Region and Australasia. Blowflies include several ubiquitous species and are primarily recognised for their medical and veterinary importance. In the Afrotropics, *Calliphora* was previously known from only two species: the native *Calliphora croceipalpis* Jaennicke and the introduced *Calliphora vicina* Robineau-Desvoidy. Two new distinctive species of *Calliphora* collected during recent fieldwork in Ethiopia are described using methods of integrative taxonomy. *Calliphora teraramma* **sp. n.** is characterised by peculiar male genitalia, with large cerci and a minute phallus. *Calliphora mesay* **sp. n.** is characterised by morphological and molecular traits, a close relative of the cosmopolitan *C. vicina*. In addition, we developed a cytochrome c oxidase subunit I (COI) barcode reference library for Palaearctic and Afrotropical *Calliphora* species, including 33 newly generated barcodes. Molecular species delimitation analyses using the software Automatic Barcode Gap Discovery (ABGD) and Assemble Species by Automatic Partitioning (ASAP), implemented through the recently developed integrative platform Spart Explorer, largely support morphological species concepts.

KEYWORDS
ABGD, Afrotropics, ASAP, *Calliphora*, cytochrome c oxidase subunit 1, DNA barcode, morphology, new species, Spart Explorer, species delimitation

INTRODUCTION

Blowflies (Calliphoridae), comprising more than 2000 described species, constitute a significant portion of the radiation of schizophoran flies and are of notable medical, veterinary and forensic importance (Parmar et al., 2025; Yan et al., 2021). The blowfly phylogeny is challenging to resolve due to the complex relationships between and within the numerous subfamilies that make up this family (Buenaventura et al., 2021; Parmar et al., 2025; Rognes, 1997; Yan et al., 2021). Recent advances in Next Generation Sequencing have facilitated high-resolution phylogenetic hypotheses for the Calliphoridae, prompting systematic revisions, including the expansion of the subfamily Calliphorinae to incorporate the former subfamilies Aphyssurinae, Melanomyinae and Toxotarsinae (Yan et al., 2021). The genus *Calliphora* Robineau-Desvoidy, the largest within Calliphorinae, includes over 100 species and has undergone numerous taxonomic revisions, including subgroup realignments and species synonymisation (Johnston et al., 2025; McDonagh & Stevens, 2011).

The genus displays a bimodal diversity pattern, peaking in the Holarctic region and Australasia. The Holarctic fauna is relatively well

documented, with 21 species identified in the Palaearctic Region (Rognes, 1991, 1997; Rognes, 2016; Schumann, 1986; Schumann & Ozerov, 1992; Tantawi et al., 2017). Due to their preference for cold environments, *Calliphora* species exhibit low diversity in tropical areas, occurring primarily at higher elevations (Lutz et al., 2018; Verves, 2005; Whitworth, 2012). In the Afrotropics, the genus was traditionally represented by just two species: the indigenous *Calliphora croceipalpis* Jaennicke, widespread across the region (Lutz et al., 2018; Pont, 1980; Zumpt, 1956), and the introduced *Calliphora vicina* Robineau-Desvoidy, recently documented in South Africa (Kelly et al., 2011; Williams & Villet, 2006). Given that Afrotropical mountainous regions are biodiversity hotspots and refuges for cold-adapted taxa (Plumptre et al., 2007), further discoveries of *Calliphora* species in high-altitude areas along the Great Rift Valley are plausible. Such habitats have previously revealed cold-adapted species within Calyptratae flies, including muscid genera *Coenosia* Meigen and *Spilogona* Schnabl (Becker et al., 1915; Pont, 1980; van Emden, 1951).

DNA barcoding, an efficient and reliable tool for species identification, has become an increasingly vital and faster alternative to traditional morphology-based bioassessment for medical and veterinary entomological research, particularly in forensic contexts (Antil et al., 2023). However, *Calliphora* representation within major genetic databases remains sparse, with only approximately 2668 records and 47 named species represented in the Barcode of Life Data System (BOLD; accessed June 2025), comprising ~47% of the genus diversity. This limits the utility of these databases, particularly for species identification and delimitation.

The primary objective of this study is to describe two newly identified *Calliphora* species collected from Ethiopian highlands and to develop a robust cytochrome c oxidase subunit I (COI) barcode reference library for Palaearctic and Afrotropical *Calliphora*. By employing integrative taxonomy, we aim to facilitate accurate species identification and support the future taxonomic revision of Old World *Calliphora* species, thus contributing valuable data for medical and veterinary entomology.

MATERIALS AND METHODS

Specimen collection and identification

Fieldwork in the Bale Mountains National Park was conducted with permission from the Ethiopian Wildlife Conservation Authority (Permit No. 79/2013). Specimen collection, focused on Oestroidea flies, was carried out by a team of four researchers, including two of the co-authors, Dinka M. D. and Szpila K., during May 2021. Adult blowflies were collected using rotting fish bait as an attractant and captured using entomological nets. Specimens were identified to the genus level using taxonomic keys by Zumpt (1956, 1965) and Rognes (1991, 1998). Terminology for adult morphology follows Rognes (1991), incorporating updates from Merz and Haenni (2000) and Zatwarnicki (1996).

Body length measurements were recorded for all type specimens (holotype and paratypes) of the new species, while other morphological features were measured exclusively from the holotype. Male terminalia were dissected and preserved as morphological vouchers. Voucher specimens are deposited at the Department of Ecology and Biogeography, Nicolaus Copernicus University, Poland. External morphology was documented using a Leica M205C stereomicroscope equipped with a high-resolution Leica DFC495 digital camera and software for focus stacking (Leica Application Suite 4.4.0). Composite images were generated from stacks of 30–35 images. Type specimens are housed in the Natural History Museum of Denmark, Copenhagen, Denmark.

DNA extraction, sequence assembly and annotation

We collected 33 specimens (32 *Calliphora* and one *Cynomya* Robineau-Desvoidy) from various localities within the Palaearctic and Afrotropical regions, including 26 males and 7 females (Tables S1 and S2). An additional 177 specimens, representing Palaearctic and Afrotropical *Calliphora* and *Cynomya*, were extracted from public repositories (NCBI, BOLD) and were included for subsequent analyses (Table S2), as detailed below. The newly collected specimens were either preserved in ethanol or maintained as pinned vouchers. Total genomic DNA was extracted from muscle tissue of one or two legs (pinned specimens) or thorax tissue (ethanol-preserved specimens) using a DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. DNA was eluted twice in 50 µL of TE buffer (100 µL total volume) and stored at −20°C. DNA quality and quantity were assessed using a Qubit 3.0 fluorometer using a dsDNA High Sensitivity Assay Kit (Life Technologies, Inc., Carlsbad, CA, USA). Gel electrophoresis on 0.5% agarose gels stained with GelRed (Biotium, Darmstadt, Germany) was used to verify DNA integrity.

The COI sequences analysed in this study were originally generated as part of a comprehensive mitogenome sequencing effort, using the genome skimming technique (Trevisan et al., 2019) and Illumina sequencing on the NovaSeq 6000 platform (Illumina, Inc., California, USA). Assembled mitogenomes were obtained from raw read data using MitoZ v3.6 (Meng et al., 2019) with the MegaHit v1.2.9 assembler (Li et al., 2015). Annotations were performed using MiTFi v0.1 (Jühling et al., 2012), tblastn (Gertz et al., 2006), Genewise (Birney et al., 2004) and infernal-1.1.1 (Nawrocki & Eddy, 2013) within MitoZ. COI sequences were extracted from the annotated mitochondrial dataset, again within MitoZ v3.6, and compared to published mitogenomes of closely related species in NCBI for length and accuracy validation. The full-length COI sequences (1536 bp) are publicly available in the BOLD v5 (Ratnasingham & Hebert, 2007) under the project DS-KEIBNCU, Barcode Library for Afrotropical and Palaearctic *Calliphora*, accessible at https://doi.org/10.5883/DS-KEIBNCU (Parmar & Szpila, 2025) and in NCBI GenBank (https://www.ncbi.nlm.nih.gov), with accession numbers provided in Table S1.

Sequence alignment and phylogenetic analysis

The newly generated sequences (33 total) were trimmed to the barcode region (~658 bp) and combined with additional barcode records (144 sequences), representing Palaearctic and Afrotropical *Calliphora* and *Cynomya*, retrieved from BOLD v5 and NCBI databases (Table S2). We selected sequences based on completeness, absence of stop codons and contamination and database availability (1–14 records per species). For species with more than 10 barcode records on BOLD, we chose 10 representative sequences at random. Synonymies were resolved according to recent taxonomic updates; for example, *Aldrichina* Townsend and *Triceratopyga* Rohdendorf are now being treated as *Calliphora* (Park et al., 2009, 2013; Rognes, 1991). The full barcode sequence database was then aligned using MAFFT v7.490 (Katoh & Standley, 2013). The alignments were manually examined to eliminate pseudogenes, nuclear mitochondrial DNA segments (NUMTs) and sequences with stop codons using Geneious Prime v.2024.0.2 (https://www.geneious.com/updates/geneious-prime-2024-0). The final alignment included 177 barcode sequences (Table S2).

Our barcode alignment was then used to generate a Maximum Likelihood (ML) phylogenetic tree using IQ-TREE v2.1.4-beta (Minh et al., 2020) with the TIM2 + F + I + G4 substitution model, which was determined as optimal by ModelFinder (Kalyaanamoorthy et al., 2017) (Based on the Bayesian information criterion (BIC)). Nodal support for the extended majority-rule consensus ML tree was assessed through ultrafast bootstrap analysis (Hoang et al., 2018) and SH-like approximate likelihood ratio tests (Guindon et al., 2010), each with 10,000 replicates. The resulting phylogeny was rooted with *Pollenia rudis* Fabricius (OQ622831.1) as the outgroup. Tree visualisation was performed in FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/), and the final figure was prepared using Inkscape 1.3.2 (available at https://inkscape.org).

Molecular species delimitation analyses

To confirm our morphological species hypotheses, we applied two complementary methods for species delimitation: Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2012) and Assemble Species by Automatic Partitioning (ASAP) (Puillandre et al., 2021) to test the efficiency of molecular barcodes in delimiting *Calliphora* and *Cynomya* species from the Palaearctic and Afrotropical regions. The dataset included a total of 177 individuals, representing 24 nominal species (Table S2). Both analyses were conducted using the recently developed Spart Explorer web platform (https://spartexplorer.mnhn.fr/), which enables integrative species delimitation through a three-step workflow: (Antil et al., 2023) species delimitation using ABGD and ASAP; (Becker et al., 1915) integration and visualisation of species partitions via a dynamic segmented bar chart; and (Birney et al., 2004) statistical comparison of alternative species hypotheses using the LIMES v2.0 (Likelihood-based Integrated Method for Evaluating Species partitions) module (Ducasse et al., 2020).

In the ABGD analysis, we set the prior intraspecific divergence (P) range from 0.001 to 0.1 with 20 steps and X-values from 1.0 to 1.2. For ASAP, the split group parameter was set to 0.01. The simple distance method (Collins et al., 2012) which has been shown to be effective in other groups of closely related species (Johnston et al., 2023; Srivathsan & Meier, 2012). The final species partitions were determined based on the lowest ASAP score (Puillandre et al., 2021) and compared with ABGD results. Additionally, to statistically evaluate congruence between molecularly inferred partitions and morphologically defined species hypotheses, we used the LIMES feature within Spart Explorer.

To supplement the Spart analysis, we also evaluated pairwise Kimura 2-parameter (K80) distances in R using the *ape::dist.dna()* function, with gaps treated via pairwise deletion. Additionally, we computed both intra- and interspecific genetic distances with the adegenet package (v2.1.10; Jombart & Bateman, 2008) in R v4.3.2 (https://www.r-project.org/), executed through Rstudio v2025.05.0 + 496 (Posit, Boston, USA).

RESULTS

Species descriptions

Calliphora teraramma sp. n.

Type material examined

Holotype: 1 Male, Ethiopia, Bale Mountains National Park, Sanetti Plateau I, 13 May 2021, 6.9094 39.9170, leg. ETH KEiB Expedition II. Paratypes: 2 Males, Ethiopia, Bale Mountains National Park, Sanetti Plateau I, 13 May 2021, 6.9094 39.9170, leg. ETH KEiB Expedition II (barcoded as KEIB_DIP_02146); 1 Male, Ethiopia, Bale Mountains National Park, Sanetti Plateau II, 13 May 2021, 6.8493 39.8917, leg. ETH KEiB Expedition II (barcoded as KEIB_DIP_02150); 1 Female, Ethiopia, Bale Mountains National Park, Dinsho, 13 May 2017, 7.1099 39.7641, leg. ETH KEiB Expedition II (barcoded as KEIB_DIP_02151).

Male (Figure 1a–g). Body length 11–13 mm ($n = 3$).

Head. Parafacial plate black with whitish-grey microtrichosity, plates broad, with the width at the level of the border with the gena similar to the width at the base of the antenna. Frons broad, tapered downwards, 0.22 head width at the ocellar triangle and 0.36 head width at the base of the antenna. Fronto-orbital plate black with whitish-grey microtrichosity except for the grey posteriormost part. Frontal vitta broad, 5 times as broad as the fronto-orbital plate at the vertex, edges parallel, slightly narrowed in mid-length, width at the lunule equal to width at the anterior ocellus, dark grey in anterior view, black in dorsal view. Scape, pedicel, first flagellomere and arista black. Ocellar triangle with a pair of antero-laterally directed setae much larger than additional ocellar setulae, and two to three pairs of postocellar setae. Inner vertical seta strong, almost straight, outer vertical seta absent. Nine to eleven pairs of strong frontal setae, pairs crossing over frontal vitta. Frontal vitta and vertex bare, except for

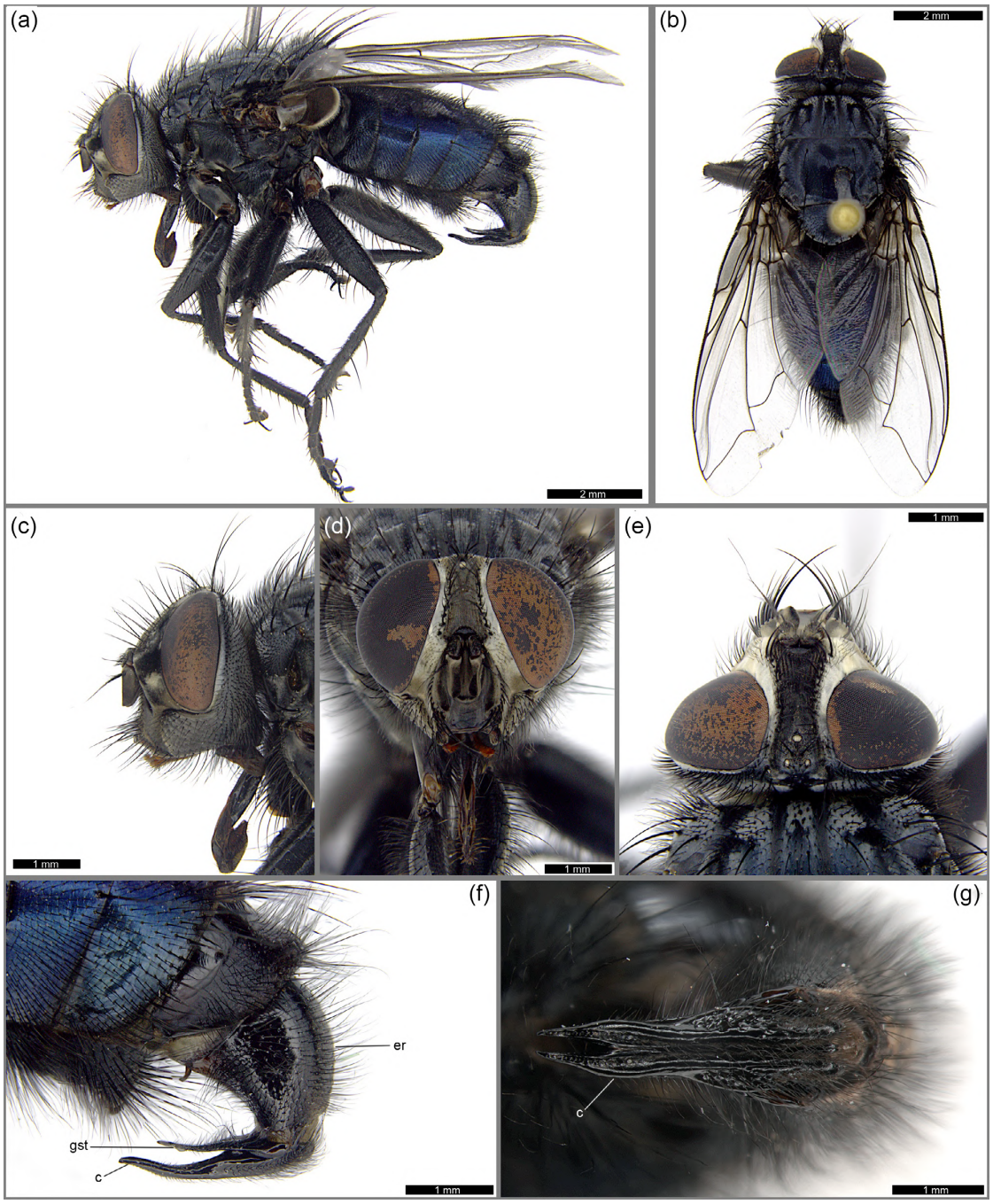

FIGURE 1 Male of *Calliphora teraramma* **sp. n.**, holotype: (a) habitus, lateral view; (b) habitus, dorsal view; (c) head, lateral view; (d) head, frontal view; (e) head, dorsal view; (f) posterior part of abdomen, lateral view; (g) cerci and gonostyli, dorsal view. c, cercus; e, epandrium; gst, gonostylus.

one pair of paravertical setae. One pair of lateroclinate orbital seta, other orbital setae absent. Parafacial plate in upper half with long, black setulae. Lunule bare. Scape and pedicel with black long setulae. Gena and postgena with long, black setulae. Postcranium with long black setulae. Antenna short, first flagellomere 1.7 times as long as pedicel and 1 times as long as the distance between the tip of the first

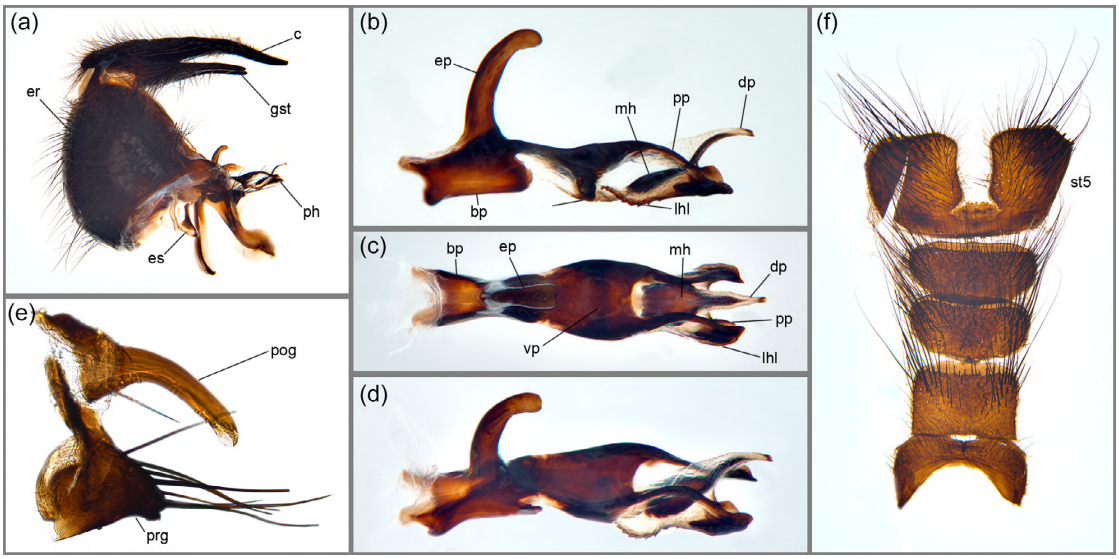

FIGURE 2 Male of *Calliphora teraramma* **sp. n.** paratype: (a) epandrial complex, lateral view; (b) phallus, lateral view: (c) phallus, dorsal view; (d) phallus, dorso-lateral view; (e) pre- and postgonite; (f) sternites of abdomen. ac, acrophallus; c, cercus; bp, basiphallus; dp, distiphallus; ep, epiphallus; es, ejaculatory sclerite; gst, gonostylus; lhl, lateral hypophallic lobe; mh, mesohypophallus; ph, phallus; pp, paraphallus; prg, pregonite; pog, postgonite; st5, fifth sternite; vp, ventral plate.

flagellomere and the lower margin of the facial plate. Arista plumose, with hairs slightly shorter ventrally, aristomeres 1 and 2 shorter than their greatest diameter, aristomere 3 widened in basal 0.2. Facial plate with low keel below antennal insertion. Vibrissa long and strong, vibrissal angle situated slightly above the lower margin of the facial plate. Subvibrissal setae numerous, long, black. Gena black, with long black setulae; height of gena 1.0 times length of the first flagellomere and 0.3 times eye height. Proboscis long, labellum 0.7 times as long as prementum, palpus yellow-orange.

Thorax. Black ground colour; covered with grey microtrichosity; scutum with shiny stripes; presutural area with four longitudinal stripes: a pair of lateral stripes between dorsocentral and posthumeral setae and a pair of inner stripes between dorsocentral and acrostichal setae; patches of shiny area also between the first and second pairs of dorsocentral setae and between the first and second pairs of acrostichal setae; anterior and posterior thoracic spiracles brownish-black; coxopleural streak absent; acrostichal setae 2 + 1, dorsocentral setae 2 + 3, interalar setae 1 + 2, one inner and one other posthumeral setae; humeral setae 3 + 1; four pairs of marginal setae on scutellum.

Legs. Black unmodified, fore tibia with two posteroventral setae, mid tibia with two anterodorsal setae.

Wing. Tegula and basicosta black, veins brownish-yellow; costal spine not developed; base of vein R_{4+5} with three setulae; distal part of vein M bent at right angle, M cell r_{4+5} open; upper calypter white with black rim, lower calypter blackish with whitish rim, upper surface of lower calypter with numerous long, black setulae.

Abdomen. Elongated and conical (dorsal view), lustrous navy blue, covered with thin, white microtrichosity; central part of tergites 3 and 4 with a longitudinal narrow lustrous line without microtrichosity. Median marginal setae on syntergite 1 + 2 and tergite 3 are absent; tergites 4 and 5 have numerous marginal setae along the posterior margin. Sternites have long setulae; sternite 5 is large and projecting downwards. Postabdomen is large and projecting.

Terminalia (Figure 2a–f). Cercus (c) black, lustrous, very long and strong, slightly curved (lateral view) and gradually tapering (lateral view) into a pointed tip (lateral and posterior view); cerci fused on 0.7 of their length; cercus with dense and long setulae in the basal half, with a gradual transition to short setulae in the distal part. Gonostylus (gst) black, straight, much shorter than cerci, pointed at the tip, with setulae along the entire length. Pregonite (prg) triangular, with seven strong setulae; postgonite (pog) slender, curved with a rounded tip and strong anterior seta. Epiphallus, basiphallus, ventral plate, paraphallus and mesohypophallus strongly sclerotised; ventral plate very broad (dorsal view); acrophallus short and directed antero-dorsally; hypophallic lobe with numerous denticles.

Female (Figure 3a–g). Differs from male in several aspects of head and abdomen morphology. Body length 11 mm ($n = 1$).

Head. Parafacial plate black with whitish-grey microtrichosity, broad, with the width at the level of the border with gena similar to the width at the base of the antenna. Frons broad, almost parallel, 0.37 head width at the ocellar triangle and 0.40 head width at the base of the antenna. Fronto-orbital plate black with whitish-grey microtrichosity except for the most posterior part. Frontal vitta broad 3.3 x as broad as fronto-orbital plate at vertex, parallel, width at lunule equal to width at anterior ocellus, black. Eight to nine pairs of strong frontal setae, pairs crossing over frontal vitta. Two pairs of proclinate orbital setae, one pair of lateroclinate orbital setae. Antenna long, first flagellomere 2.6× as long as pedicel and 2.0× as long as the distance

FIGURE 3 Female of *Calliphora teraramma* **sp. n.**, paratype: (a) habitus, lateral view; (b) habitus, dorsal view; (c) head, lateral view; (d) head, frontal view; (e) head, dorsal view; (f) fifth tergite of abdomen, lateral view; (g) fifth tergite of abdomen, dorsal view.

between the tip of the first flagellomere and the lower margin of the facial plate. Height of gena 0.84 x length of first flagellomere and 0.35× eye height.

Abdomen. Oval in dorsal view, lustrous navy blue, covered with thin, white microtrichosity like in male but central part of tergites 3 and 4 without a longitudinal narrow lustrous line without microtrichosity. Fifth tergite with dense strong setae at the posterior margin and dorsal surface.

Biology

Specimens were attracted to fish bait and human faeces in mountainous open habitats.

DNA barcoding

All barcoded specimens of *C. teraramma* (males and female) were grouped together in the phylogenetic and species delimitation analyses (Figure S1; Figure S2). Pairwise Kimura 2-parameter (K80) distances revealed clear genetic differentiation between *Calliphora teraramma* and all other analysed species (*Calliphora* spp. and *Cynomya mortuorum* Linnaeus) (Table S3). The smallest interspecific distance between *C. teraramma* in comparison to other *Calliphora* and *Cynomya* species was 5.26% and 7.9%, respectively. While the maximum intraspecific distance observed in *C. teraramma* was 1.52%, leading to a barcode gap ranging from a minimum of 3.74% to a maximum of 6.38%, supporting its status as a well-defined species under the DNA barcoding criterion (Hebert et al., 2003).

Etymology

Named from the Amharic word for "mountainous" (by mountainous distribution) – "ካሊፎራ ተራራማ" with Latin transliteration "teraramma".

Remarks

Identifying specimens of *C. teraramma* **sp. n**. using the keys provided by Zumpt (1956, 1965) and Rognes (1991, 1998) leads, despite a few exceptions (one pair of postsutural acrostichal setae), to genus *Calliphora*. The species is distinctly new because of the peculiar form of the male genital apparatus: strong cercus, relatively short gonostylus, short phallus with a broad ventral plate in dorsal view and upraised epiphallus.

Calliphora mesay **sp. n.**

Type material examined

Holotype: 1 Male, Ethiopia, Bale Mountains National Park, Sanetti Plateau I, 13 May 2021, 6.9094 39.9170, leg. ETH KEiB Expedition II (barcoded as KEIB_DIP_02147). Paratypes: 1 Male, Ethiopia, Bale Mountains National Park, Sanetti Plateau I, 13 May 2021, 6.9094 39.9170, leg. ETH KEiB Expedition II; 1 Female, Ethiopia, Bale Mountains National Park, Sanetti Plateau I, 13 May 2021, 6.9094 39.9170 (barcoded as KEIB_DIP_02148).

Male (Figure 4a–g). Body length 8–10 mm ($n = 2$).

Head. Parafacial plate black with whitish-grey microtrichosity, plates very broad, with the width at the level of the border with gena similar to the width at the base of the antenna. Frons narrow, widened downwards, 0.11 head width at the ocellar triangle and 0.37 head width at the base of the antenna. Fronto-orbital plate black with whitish-grey microtrichosity except for the grey posteriormost part. Frontal vitta broad 10 times as broad as the fronto-orbital plate at the vertex, strongly widened anteriorly, with the width at the base of the antenna 2 times the width at the ocellar triangle, black. Scape, pedicel, first flagellomere and arista black. Ocellar triangle with a pair of anteriorly directed setae larger than additional ocellar setulae, and two to three pairs of postocellar setae. Inner vertical seta strong, almost straight; outer vertical seta absent. Eleven to thirteen pairs of strong frontal setae, pairs crossing over the frontal vitta. Frontal vitta and vertex bare, except for one pair of paravertical setae. Orbital setae absent. Parafacial plate in the upper half with long, black setulae. Lunule bare. Scape and pedicel with long black setulae. Gena and postgena with long, black setulae. Postcranium with long black setulae. Antenna long, first flagellomere 3.2 times the pedicel length and 1.5 times the distance between the tip of the first flagellomere and the lower margin of the facial plate. Arista plumose, aristomere 2 length similar to its maximum diameter, aristomere 1 shorter than its maximum diameter, aristomere 3 widened in the basal 0.3. Facial plate with a distinct keel below the antennal insertion. Vibrissa long and strong, vibrissal angle situated slightly above the lower margin of the facial plate. Subvibrissal setae numerous, long, black. Gena black, with long black setulae; height of the gena 1.1 times the length of the first flagellomere and 0.4 times eye height. Proboscis long, labellum 0.7 times as long as the prementum, palpus yellow-orange.

Thorax. Black ground colour; covered with grey microtrichosity; scutum with shiny stripes; presutural area with four longitudinal stripes: a pair of lateral stripes between dorsocentral and posthumeral setae and a pair of inner stripes between dorsocentral and acrostichal setae; anterior and posterior thoracic spiracles brownish-black; coxopleural streak absent; acrostichal setae $2 + 3$, dorsocentral setae $3 + 3$ (the first presutural pair less strong), interalar setae $1 + 2$, one inner and one outer posthumeral setae; humeral setae $3 + 1$; four pairs of marginal setae on scutellum.

Legs. Black without particular modification, fore tibia with two posteroventral setae, mid tibia with three anterodorsal setae.

Wing. Tegula and basicosta black, veins blackish-brown; costal spine not developed; base of vein R_{4+5} with 6 to 7 setulae; distal part of vein M bent at an acute angle (a little less than 90 degree), M cell r_{4+5} open; upper calypter blackish with a black rim, lower calypter black with a whitish rim, upper surface of the lower calypter with numerous long, black setulae.

Abdomen. Rounded (dorsal view), lustrous navy blue, covered with thin, white microtrichosity; the central part of tergites 3 and 4 has a longitudinal narrow lustrous line without microtrichosity. Median marginal setae on syntergite $1 + 2$ and tergite 3 are absent; tergites 4 and 5 have numerous marginal setae along the posterior margin. Sternites have long setulae; the sternite not projecting. Postabdomen small.

Terminalia (Figure 5a–e). Cercus (c) black, long and slender, straight (lateral view) and gradually tapering (lateral view) into a pointed tip (lateral and posterior view); cerci fused in 0.5 of their length; cercus with long setulae in the basal half, gradually transitioning to shorter setulae in the distal part. Gonostylus (gst) brown, straight, slightly shorter than cerci, rounded at the tip, with setulae along the whole length. Pregonite (prg) rectangular with a long anterodorsal extension, with four strong setulae; postgonite (pog) slender, curved with a rounded tip and small anterior seta. Epiphallus, basiphallus, ventral plate, paraphallus, mesohypophallus and hypophallic processes sclerotised; epiphallus long and slender, ventral plate narrow (dorsal view); acrophallus directed anteriorly; hypophallic lobe with numerous denticles.

Female (Figure 6a–e). Differs from male in several aspects of head and abdomen morphology. Body length 9–10 mm ($n = 2$).

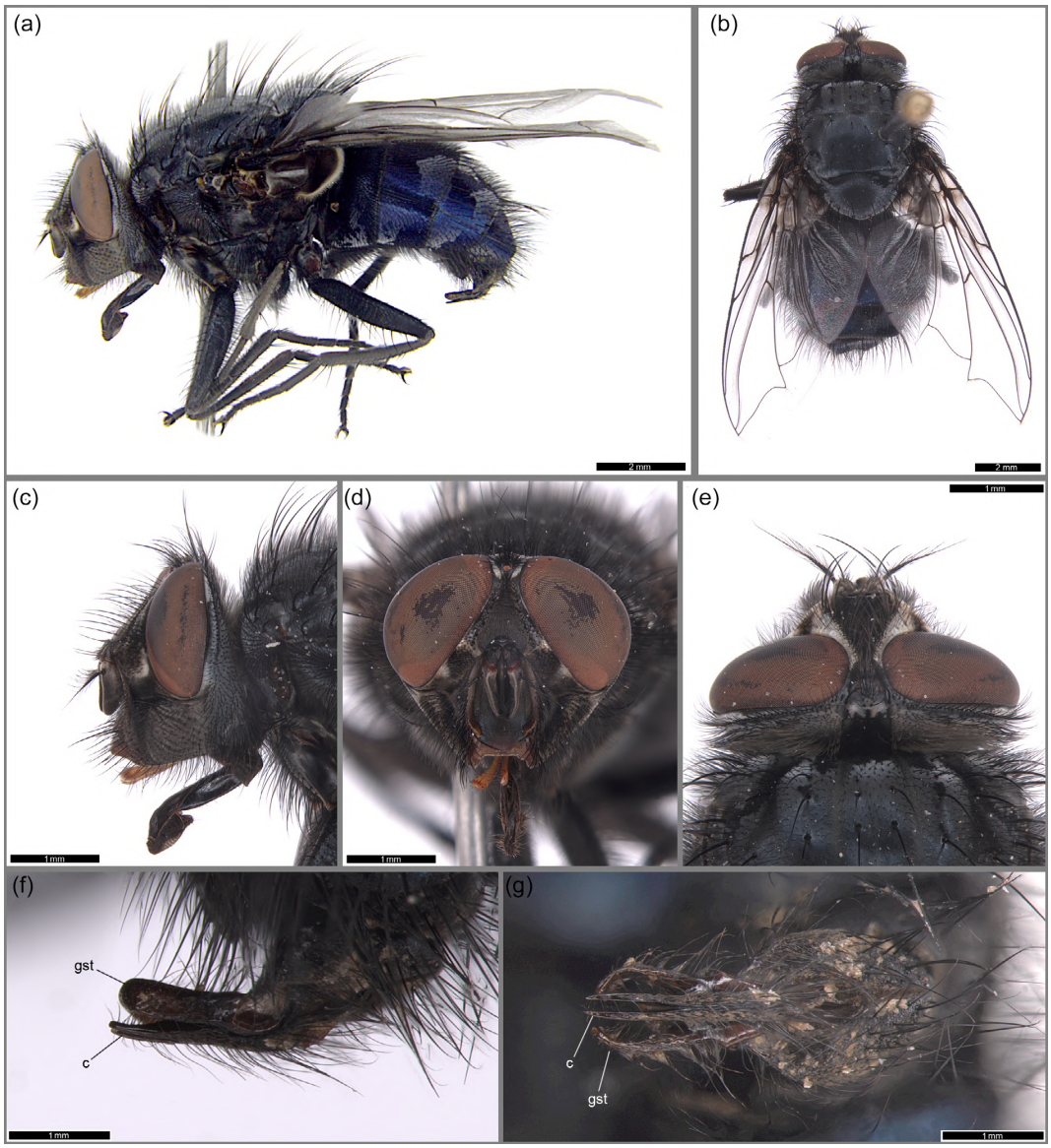

FIGURE 4 Male of *Calliphora mesay* **sp. n.**, holotype: (a) habitus, lateral view; (b) habitus, dorsal view; (c) head, lateral view; (d) head, frontal view; (e) head, dorsal view; (f) cerci and gonostyli, lateral view; (g) cerci and gonostyli, dorsal view. c, cercus; e, epandrium; gst, gonostylus.

Head. Parafacial plate black with weak whitish-grey microtrichosity, plates broad, with the width at level of border with gena similar to the width at the base of antenna. Frons broad, almost parallel, 0.37 head width at the ocellar triangle and 0.42 head width at the base of antenna. Fronto-orbital plate black with whitish-grey microtrichosity, including the most posterior part. Frontal vitta broad 3.3 times as broad as fronto-orbital plate at vertex, parallel, width at lunule equal to width at anterior ocellus, black. Eight to nine pairs of strong frontal setae, pairs crossing over frontal vitta. Two pairs of proclinate orbital setae, one pair of lateroclinate orbital setae. Antenna long, first flagellomere 2.6 times as long as pedicel and 2.0 times as long as distance between tip of first flagellomere and lower margin of facial plate. Height of gena 0.84 times length of first flagellomere and 0.35 times eye height.

Abdomen. Oval in dorsal view, lustrous navy blue, covered with thin, white microtrichosity. Fourth and fifth tergites with marginal setae at the posterior margin. Fifth tergite with numerous discal setae on the dorsal surface. Ovipositor not dissected.

Biology

Specimens were attracted to fish bait and human faeces in mountainous open habitats.

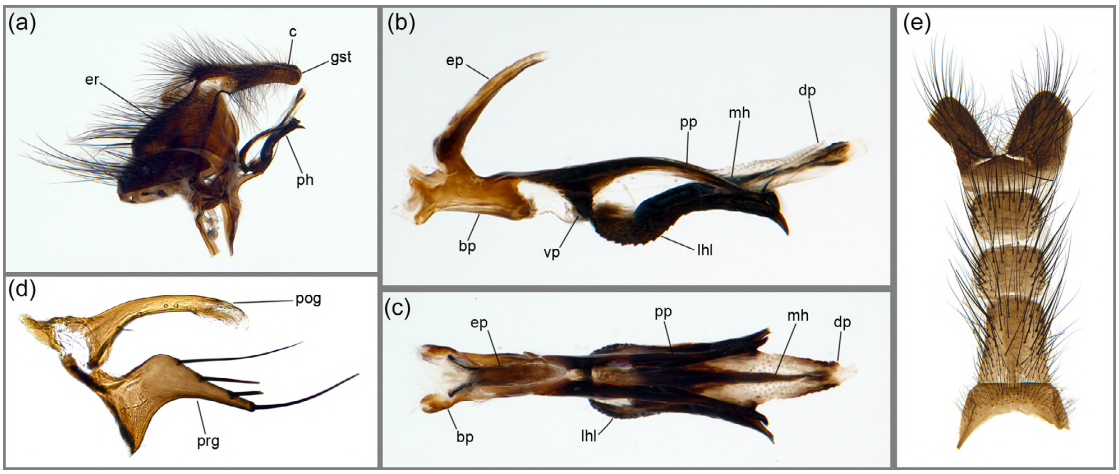

FIGURE 5 Male of *Calliphora mesay* **sp. n.**, paratype: (a) Epandrial complex, lateral view; (b) phallus, lateral view; (c) phallus, dorsal view; (d) pre- and postgonite; (e) sternites of abdomen. ac, acrophallus; c, cercus; bp, basiphallus; dp, distiphallus; ep, epiphallus; es, ejaculatory sclerite; gst, gonostylus; lhl, lateral hypophallic lobe; mh, mesohypophallus; ph, phallus; pp, paraphallus; prg, pregonite; pog, postgonite; vp, ventral plate.

DNA barcoding

All barcoded specimens of *C. mesay* (male and female) clustered together in phylogenetic analysis and formed a sister group to the clade containing *Calliphora vicina* Robineau-Desvoidy (Figure S1), while species delimitation analyses by both ABGD and ASAP could not clearly resolve *C. mesay* as a distinct species (Figure S2). The interspecific distance between *C. mesay* and *C. vicina* ranged from 0.6% to 1.2%, which increased to 0.85%–0.91% when the full COI region (1536 bps) was analysed, and to 0.80%–0.83% when all thirteen protein-coding genes (PCGs) were used. These results provide robust molecular support for distinguishing *C. mesay* from *C. vicina*, complementing the morphological diagnostic traits described earlier.

Etymology

Named from the Amharic word for "similar" (by similarity to *C. vicina*) – "ካሊፎራ ሕሳይ" with Latin transliteration "mesay".

Remarks

Identifying specimens of *C. mesay* **sp. n.** using the keys provided by Zumpt (1956, 1965) and Rognes (1991, 1998) leads to genus *Calliphora*, and the morphology of the male genital apparatus points to a close relation to *C. croceipalpis* and *C. vicina*. The species differs distinctly from *C. vicina* in the following character states: dark parafacial and fronto-orbital plates, dark face and genal dilation, dark anterior thoracic spiracle and black basicosta. *Calliphora mesay* **sp. n.** may be easily separated from co-occurring *C. croceipalpis* by the following: broad frons in males, very broad parafacial plates in both sexes, presence of long and dense setulae on parafacial plates and dark anterior thoracic spiracles (Martínez-Sánchez et al., 2024).

Phylogenetic analysis

Maximum likelihood inference using IQ-TREE revealed well-supported clades (>80% UFBS), largely consistent with morphological species hypotheses (Figure S1). Some species groups, such as *Calliphora alaskensis* Shannon and *Calliphora subalpina* Ringdahl, were split into two clades. Notably, one *Calliphora loewi* Enderlein specimen sequenced in this study clustered as a sister to *Calliphora sinensis* Aubertin, deviating from other *C. loewi* specimens, suggesting this specimen may represent a genetically unique individual or morphologically cryptic hybrid as observed in other blowfly species (Croft et al., 2024).

Consistent with previous studies (Chen et al., 2016), *Calliphora chinghaiensis* Van et Ma was closely related to *Calliphora grahami* Aldrich, while *Calliphora zaidamensis* Fan formed a clade with *Calliphora uralensis* Villeneuve. Although the identification of *C. zaidamensis* was based on expert taxonomic assessment (Prof. Yuriy Verves), the specimen was in poor condition, and a degree of uncertainty cannot be ruled out. Nevertheless, the close phylogenetic relationship between *C. zaidamensis* and *C. uralensis* is supported by their reported morphological affinities (Peris & González-Mora, 1989), suggesting that the observed clustering may reflect genuine evolutionary relatedness. The newly described *C. teraramma* **sp. n.** formed a distinct clade with strong support (>80% UFBS), confirming its status as a separate Afrotropical species. Similarly, *C. mesay* **sp. n.** from Ethiopia formed a close relationship with the cosmopolitan *C. vicina*, congruent with the morphological similarities (genital apparatus) between these species.

Species delimitation analysis

Our final COI sequence database included 177 sequences representing 24 described species (including the two newly described species). Species delimitation analyses of this database with both ABGD and ASAP methods yielded congruent results. ABGD analysis resulted in initial partitions with 22 species regardless of the applied relative gap width ($X = 1.0$, 1.1 or 1.2) across all prior intraspecific divergence values (p) ranging from 0.0001 to 0.0012 (Table S4). For $p > 0.0012$, species numbers rapidly decreased to one. Recursive partitioning

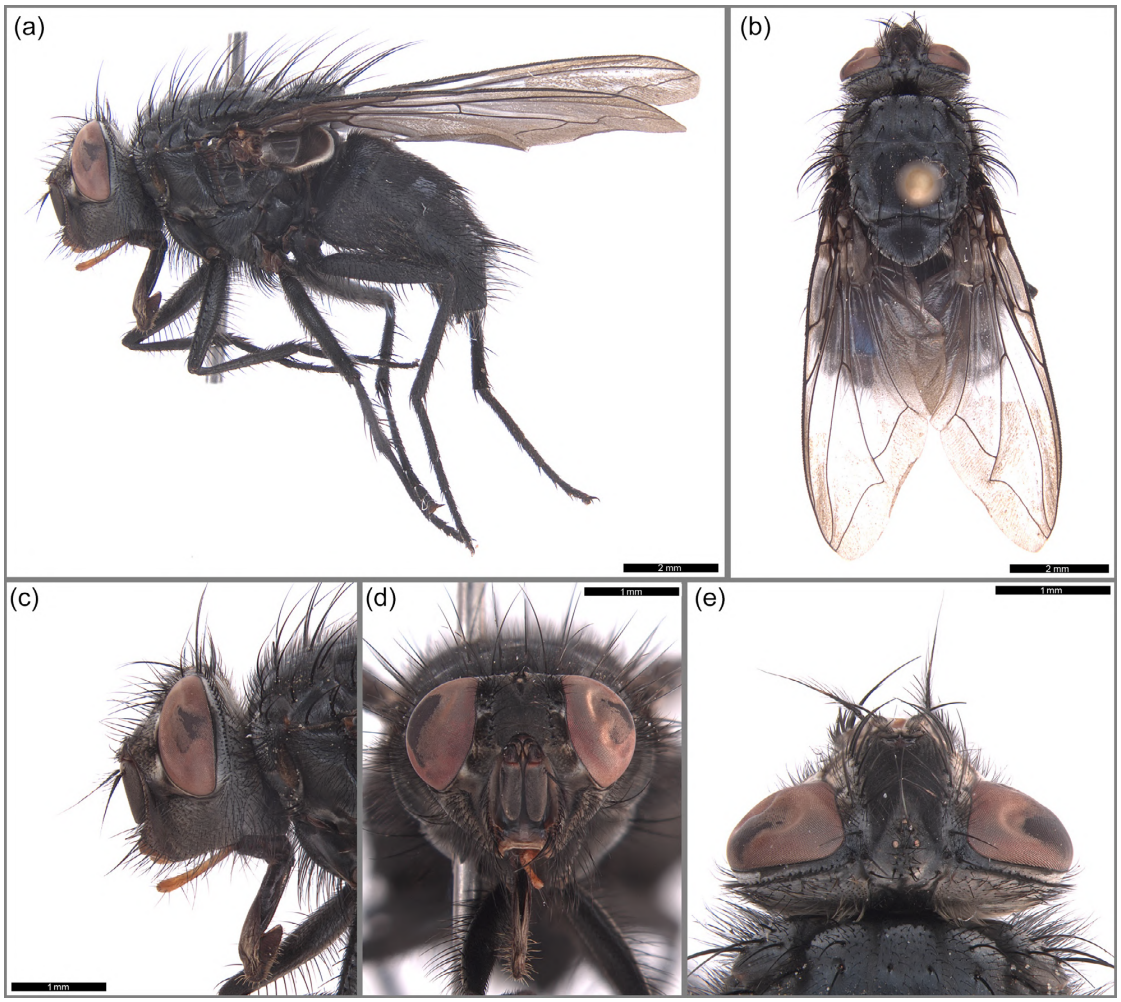

FIGURE 6 Female of *Calliphora mesay* **sp. n.**, paratype: (a) habitus, lateral view; (b) habitus, dorsal view; (c) head, lateral view; (d) head, frontal view; (e) head, dorsal view.

identified 25 species ($p = 0.0001–0.0012$). Species delimitation using ASAP also identified 22 species groups as the optimal partition scheme, in agreement with the ABGD analysis (ASAP score = 3.0; $p = 0.012$; W-rank = 0.000051; and threshold distance = 0.010). The second-best partitioned scheme suggested 13 species groups (ASAP score = 4.5; $p = 0.11$; W-rank = 0.000017; and threshold distance = 0.026) (Table S4). Of the 22 delimited species in both analyses, 18 corresponded with described morphospecies. Several discrepancies were observed between the species described using morphology and the molecularly delimited groups, particularly among species with overlapping morphological characters: (Antil et al., 2023) *Calliphora loewi*, *C. sinensis* and *C. subalpina* were grouped into a single species cluster; (Becker et al., 1915) *C. alaskensis* specimens were divided into two distinct clusters; (Birney et al., 2004) specimens of *C. mesay* **sp. n.** were grouped with *C. vicina*; (Buenaventura et al., 2021) *C. rohdendorfi* individuals from Russia and Poland formed separate clusters, potentially reflecting geographic divergence; (Chen et al., 2016) *C. chinghaiensis* grouped with *C. grahami*, as previously noted by Chen et al. (2016); (Collins et al., 2012) *C. uralensis* was split into two subsets, one of which included a specimen of *C. zaidamensis*, likely due to shared morphological traits (Peris & González-Mora, 1989).

All three specimens of the newly described *Calliphora teraramma* **sp. n.** formed a single cluster that was strongly delimited from other *Calliphora* and *Cynomya* species clusters. Alternatively, *C. mesay* **sp. n.** clustered with its morphologically similar sister species *C. vicina* (Figure S2).

DISCUSSION

Adult flies of the genus *Calliphora* are readily recognisable due to their large size, diurnal activity and tendency to visit substrates like human

and animal faeces or vertebrate carrion (Rognes, 1991; Saloña-Bordas et al., 2020). Therefore, despite the relatively poor recognition of the Afrotropical Calliphoridae (Lutz et al., 2018; Pont, 1980; Szpila et al., 2024), the discovery of two new *Calliphora* species from the Bale Mountains is noteworthy.

Low COI divergence but strong morphological support for *Calliphora mesay* sp. n

Our morphological and molecular data unequivocally indicate a close relationship between *Calliphora mesay* **sp. n**. and the cosmopolitan *C. vicina*, whose natural range extends to southern Egypt (Pont, 1980; Rognes, 1991; Schumann, 1986). Although the interspecific divergence in the standard COI barcode region are minimal (0.6%–1.2%, differs by 8 bps) and may not meet molecular delimitation criteria (Meier et al., 2006), they can be distinguished by 16 nucleotide differences across the full COI gene and 98 differences across all protein-coding mitochondrial genes.

Although molecular data alone might not justify separate species status due to the limited COI divergence, the combination of consistent morphological differences—particularly in external coloration and head and thoracic traits—and diagnostic nucleotide differences across multiple mitochondrial regions reliably separates *C. mesay* **sp. n**. from *C. vicina* and further supports the recognition of *C. mesay* **sp. n**. as a valid and distinct taxon, new to science. Similar instances of limited COI resolution between morphologically distinct sister species have been documented within Calliphoridae (Johnston et al., 2025; Sonet et al., 2012; Wells et al., 2007; Whitworth et al., 2007; Williams et al., 2016). In our study, the interspecific distance between *Calliphora mesay* and *C. vicina* is approximately 1–3% lower than what has typically been observed in other closely related species within the family (Johnston et al., 2025; Sonet et al., 2012; Whitworth et al., 2007). The low interspecific divergence (0.6%–1.2%) between *Calliphora mesay* **sp. n**. and *C. vicina* likely reflects the limitations of mitochondrial DNA, which often lacks resolution for recently diverged or morphologically similar species (Kapoor et al., 2023). Low mtDNA variation is not uncommon in conspecifics, and as such, we emphasise the importance of combining mitochondrial and nuclear DNA with broad sampling to unambiguously delimit species boundaries (Nelson et al., 2007; Tantawi et al., 2010; Williams et al., 2008; Williams & Villet, 2013).

Distinctive morphological and genetic traits of *Calliphora teraramma* sp. n

In contrast to *C. mesay* **sp. n**., the newly described *C. teraramma* **sp. n**. is both genetically and morphologically unique compared to other *Calliphora* species. The male possesses a unique combination of features, including a single pair of acrostichal setae and large long cerci, similar to the genus *Cynomya*. However, *C. teraramma* **sp. n**. can be distinguished from both *Cynomya* and *Calliphora* through the presence of well-developed gonostyli and, above all, the distinctive phallus structure—characterised by a small size, fully sclerotised epiphallus, broad ventral plate (in dorsal view), broad and strongly sclerotised mesohypophallus (dorsal view), and short, upraised distiphallus (Hall, 1948; Rognes, 1991). Additionally, the gonostylus is considerably shorter than the cercus, and the pregonite is of a regular triangular shape, without an elongated process present in other *Calliphora* species (Rognes, 1991, 1997; Schumann & Ozerov, 1992; Tantawi et al., 2017; Whitworth, 2012). It should be noted that a broad ventral plate is also present in *Calliphora genarum* Zetterstedt and *Calliphora grunini* Schumann & Ozerov, and in the former species the distiphallus is short and upward-directed (Grunin, 1970; Rognes, 1991).

The female of *C. teraramma* **sp. n**. also displays unique characteristics, such as thick and long setae projecting beyond the posterior edge of the fifth tergite margin. Strong setae on the fifth tergite are observed in some *Calliphora* species (Rognes, 1991; Tantawi et al., 2017; Whitworth, 2012). They are arranged in a regular row on the edge of the tergite and can be thick and densely arranged, as in *Calliphora stelviana* Zetterstedt and *C. chinghaiensis*, or somewhat more delicate and sparsely arranged, as in *C. subalpina* or *Calliphora montana* Shannon. However, in *C. stelviana*, *C. chinghaiensis*, *C. subalpina* and *Calliphora montana*, the modified setae never extend beyond the edge of the tergite like in *C. teraramma* **sp. n**., which makes this another unique character state of this species.

Reflections on species delimitation and phylogenetic uncertainty

Our species delimitation analyses successfully delineated morphospecies from Palaearctic and Afrotropical *Calliphora* and *Cynomya* taxa. Although instances of splitting and lumping of morphospecies were observed, most described species were accurately delimited. The discordant placement of *C. loewi* (KEIB_DIP_01571) and *C. sinensis* (MT017707.1) may indicate misidentification, sequencing error or cryptic species. Either of these scenarios could hinder accurate phylogenetic resolution using mitochondrial loci, particularly when only a single or a few individuals of each species are included. We recommend future studies exercise caution when using mitochondrial DNA alone to analyse small datasets of *Calliphora*, as without robust taxon sampling, anomalous individuals cannot be reliably detected. In contrast to this, the over-splitting of morphospecies encountered here might also be caused by geographic isolation between two populations (Johnston et al., 2023), resulting in genetically distinct groups as observed within *C. subalpina*, sampled from Poland, Germany and Russia.

AUTHOR CONTRIBUTIONS

Drashti R. Parmar: Conceptualization; investigation; writing – original draft; writing – review and editing; data curation; formal analysis; visualization; validation; methodology; software. **Nikolas P. Johnston:** Writing – review and editing. **Mergi Daba Dinka:** Writing – review and editing. **Krzysztof Szpila:** Project administration; supervision;

resources; conceptualization; funding acquisition; investigation; writing – original draft; writing – review and editing; data curation; methodology; validation; visualization.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All sequence data generated and analysed in this study are publicly available. The mitochondrial COI sequences have been deposited in NCBI GenBank, https://www.ncbi.nlm.nih.gov, and are accessible under accession numbers provided in Table S1. The COI barcode dataset is available in the Barcode of Life Data System (BOLD) under the project DS-KEIBNCU, Barcode Library for Afrotropical and Palaearctic *Calliphora*, accessible at https://doi.org/10.5883/DS-KEIBNCU (Parmar & Szpila, 2025).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Fig. S1. Maximum likelihood tree for *Calliphora* and *Cynomya* based on mitochondrial cytochrome 1 (COI), using IQ-TREE under BIC-based model selection. The dataset includes COI barcodes of 178 taxa, including Polleniidae as an outgroup. Red circles indicate branches with >80% bootstrap support. Specimens barcoded in this study are highlighted in blue. The clades with two newly described species are marked in green. For clarity, voucher codes or Genbank accession numbers are included along with the species with more than one specimen.

Fig. S2. Species delimitation results for COI sequences (177 individuals, 24 nominal species) analysed using two distance-based methods (ABGD, ASAP) in Spart Explorer. Coloured bars represent species groups delimited by each method, with individual counts shown numerically. The ASAP score (lower value) and inferred species number (upper value) are displayed above the bars. The cladogram provides phylogenetic support for the delimitation patterns.

Table S1. Supplementary Tables

Data S1. Structured reflexivity statement.

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Table S1. Summary of specimens and accession data sequenced in this study. Newly described species and corresponding specimens' data are highlighted in bold.

SI No.	Species	Voucher ID	Location	Latitude	Longitude	Sex	GenBank accession number	BOLD Accession number
1	*Calliphora alaskensis* (Shannon, 1923)	KEIB_DIP_02099	West Vancouver, BC, Canada	49.37	-123.24	Male	PQ593972	KEIB002-24
2	*Calliphora calliphoroides* (Rohdendorf, 1931)	KEIB_DIP_02122	Nakajima, Sakura Ward, Saitama, Japan	35.86	139.62	Male	PQ594007	KEIB014-24
3		KEIB_DIP_02102	Kabansky District, Buryatia, Russia	51.38	105.17	Male	PQ593973	KEIB004-24
4	*Calliphora croceipalpis* (Jaennicke, 1867)	KEIB_DIP_02043	Entoto Park, Addis Ababa, Ethiopia	9.08	38.73	Male	PQ594002	KEIB049-24
5	*Calliphora genarum* (Zetterstedt, 1838)	KEIB_DIP_02109	Kabansky District, Buryatia, Russia	51.40	105.07	Female	PQ593975	KEIB006-24
6	*Calliphora grahami* (Aldrich, 1930)	KEIB_DIP_02110	Kangwon, North Korea	38.68	128.18	Male	PQ593976	KEIB007-24
7	*Calliphora himalayana* (Kurahashi, 1994)	KEIB_DIP_02113	Botapathri Woods, Forest Block, Gulmarg, India	34.08	74.34	Male	PQ593978	KEIB009-24
8	*Calliphora latifrons* (Hough, 1899)	KEIB_DIP_01711	Short Canyon, Sierra Sands Unified School District, CA, USA	35.70	-117.88	Male	PQ593991	KEIB035-24
9	*Calliphora loewi* (Enderlein, 1903)	KEIB_DIP_01571	Gmina Janów, Siedlec, Poland	50.69	19.41	Male	PQ594008	KEIB039-24
10		KEIB_DIP_02118	Kabansky District, Buryatia, Russia	51.38	105.17	Male	PQ654167	KEIB012-24
11	***Calliphora mesay* sp. n.**	**KEIB_DIP_02147**	**Bale Mountains NP, Sanetti Plateau I, Fasile Angeso, Ethiopia**	**6.90**	**39.91**	Male	**PQ593985**	**KEIB029-24**
12		**KEIB_DIP_02148**	**Bale Mountains NP, Sanetti Plateau I, Fasile Angeso, Ethiopia**	**6.90**	**39.91**	Female	**PQ593986**	**KEIB030-24**
13	*Calliphora nigribarbis* (Vollenhoven, 1863)	KEIB_DIP_02123	Himonya, Meguro City, Tokyo, Japan	35.61	139.68	Male	PQ428994	KEIB015-24
14	*Calliphora rohdendorfi* (Grunin, 1966)	KEIB_DIP_02018	Przełęcz Łaszczowa, Bardo, Poland	50.47	16.73	Male	PQ593995	KEIB041-24
15		KEIB_DIP_02130	Gorod Gelendzhik, Krasnodar Krai, Russia	44.53	38.56	Female	PQ593980	KEIB017-24

16	*Calliphora* sp. n. 3 (Unknown)	KEIB_DIP_02070	Fajã da Ovelha, Madeira, Portugal	32.80	-17.16	Female	PQ593971	KEIB001-24
17	*Calliphora splendens* (Macquart, 1839)	KEIB_DIP_02131	Guía de Isora, Santa Cruz de Tenerife, Spain	28.26	-16.74	Male	PQ428995	KEIB018-24
18	*Calliphora stelviana* (Brauer & Bergenstamm, 1891)	KEIB_DIP_02132	Vargvägen 3, 830 19 Storlien, Sweden	63.31	12.10	Male	PQ428996	KEIB019-24
19	*Calliphora subalpina* (Ringdahl, 1931)	KEIB_DIP_01463	89143 Blaubeuren, Germany	48.43	9.72	Male	PQ594009	KEIB048-24
20		KEIB_DIP_02135	Gmina Czorsztyn, Poland	49.39	20.39	Male	PQ593981	KEIB020-24
21		KEIB_DIP_02136	Kabansky District, Buryatia, Russia	51.40	105.07	Male	PQ593982	KEIB021-24
22		KEIB_DIP_02137	Kabansky District, Buryatia, Russia	51.33	104.68	Male	PQ428997	KEIB022-24
23	***Calliphora teraramma* sp. n.**	**KEIB_DIP_02146**	**Bale Mountains NP, Sanetti Plateau II, Shedem, Ethiopia**	**6.84**	**39.89**	Male	**PQ593984**	**KEIB028-24**
24		**KEIB_DIP_02150**	**Bale Mountains NP, Sanetti Plateau II, Shedem, Ethiopia**	**6.84**	**39.89**	Male	**PQ593987**	**KEIB031-24**
25		**KEIB_DIP_02151**	**Bale Mountains NP, Dinsho, Ethiopia**	**7.10**	**39.76**	Female	**PQ593988**	**KEIB032-24**
26	*Calliphora terraenovae* (Macquart, 1851)	KEIB_DIP_02138	Lind, Washington, USA	46.97	-118.61	Male	PQ594006	KEIB023-24
27	*Calliphora uralensis* (Villeneuve, 1922)	KEIB_DIP_01672	Priuralsky District, Yamalo-Nenets Autonomous Okrug, Russia	66.8	65.8	Male	PQ593993	KEIB038-24
28		KEIB_DIP_02140	Suwalki Landscape Park, Gmina Jeleniewo, Poland	54.22	22.81	Male	PQ594004	KEIB024-24
29	*Calliphora vicina* (Robineau-Desvoidy, 1830)	KEIB_DIP_02142	Kerman, Kerman Province, Iran	30.19	57.43	Male	PQ593983	KEIB025-24
30		KEIB_DIP_01527	Święta Katarzyna, Poland	50.90	20.88	Male	PQ593989	KEIB033-24
31	*Calliphora vomitoria* (Linnaeus, 1758)	KEIB_DIP_01540	Gmina Nowa Słupia, Jeleniów, Poland	50.83	21.10	Female	PQ593990	KEIB034-24
32	*Calliphora zaidamensis* (Fan, 1986)	KEIB_DIP_02145	Lake Teletskoye, Altai Republic, Russia	51.78	87.25	Female	PQ594005	KEIB027-24
33	*Cynomya mortuorum* (Linnaeus, 1761)	KEIB_DIP_02028	Płock, Poland	52.56	19.67	Male	PQ664986	KEIB068-24

Table S2. COI Barcode library specimens and accession numbers. Library specimen identifiers in bold are sequenced in this study.

Species	No. of COI Sequences	Sequence Identifiers on BOLD	GenBank Accession	Sex
Calliphora alaskensis	10	**KEIB002-24**	**PQ593972**	Male
		BBDCP328-10	JF868699	NA
		BBDEC708-09	HM412265	NA
		BNNR008-11	JN263392	Female
		BNNR142-11	KX422285	Male
		BNNR143-11	KX422300	Male
		BNNR146-11	KX422297	Female
		BNNR147-11	KX422284	Female
		BNNR219-15	KX422281	Female
		GMOAB003-21	----------	NA
Calliphora calliphoroides	16	**KEIB014-24**	**PQ594007**	Male
		KEIB004-24	**PQ593973**	Male
		----------	MK893471.1	NA
		----------	MN131048	NA
		----------	MT017731	NA
		GBDP30385-19	KY001911	NA
		GBDP7925-09	EU880179	NA
		GBDP7926-09	EU880178	NA
		GBDP7927-09	EU880177	NA
		GBDP7928-09	EU880176	NA
		GBMIN53180-17	KY001913	NA
		GBMIN53181-17	KY001912	NA
		GBMIN53182-17	KY001910	NA
		GBMIN53183-17	KY001909	NA
		GBMNF24053-22	LC684786	NA
		GBMNF24054-22	LC684787	NA
Calliphora chinghaiensis	1	----------	KT936147.1	NA
Calliphora croceipalpis	1	**KEIB049-24**	**PQ594002**	Male
Calliphora genarum	11	**KEIB006-24**	**PQ593975**	Female
		CCHAR10616-19	MN669065	NA
		CCHAR6795-19	MN674291	NA
		CCHAR9853-19	MN680196	NA
		DCHAR1987-19	MN683291	NA
		DCHAR2581-19	MN670433	NA
		DCHAR2701-19	MN669052	NA
		DCHAR3166-19	MN666408	NA
		FCHAR3306-19	----------	NA
		MCHAD589-19	----------	NA
		CCHAR10651-19	MN666999	NA
Calliphora grahami	12	**KEIB007-24**	**PQ593976**	Male
		----------	KP872701.1	NA

		BNNR215-15	KX422286	Male
		GBDP30113-19	KM497305	NA
		GBDP30115-19	KY031811	NA
		GBMIN53122-17	KM497304	NA
		GBMIN53123-17	KM497307	NA
		GBMND46116-21	MW564246	NA
		GBMNF24041-22	LC684777	NA
		GBDP15405-14	KC249688	NA
		GBDP7922-09	EU880182	NA
		GBMNA9795-19	KP872701	NA
Calliphora himalayana	1	**KEIB009-24**	**PQ593978**	Male
Calliphora latifrons	11	**KEIB035-24**	**PQ593991**	Male
		BBDIV720-12	----------	NA
		BNNR216-15	KX422290	Male
		DIPUS338-10	HQ945031	NA
		DIPUS343-10	HQ945033	NA
		SSDWA5702-15	MG120137	NA
		BBDCQ711-10	JN291490	NA
		SSDWA5695-15	MG115390	NA
		BBDCP327-10	JF868698	NA
		DIPUS344-10	HQ945034	NA
		GBDP0589-06	AF295557	NA
Calliphora loewi	12	**KEIB039-24**	**PQ594008**	Male
		KEIB012-24	**PQ654167**	Male
		AMCAF543-19	----------	NA
		AMCAJ155-19	----------	NA
		AMCAL006-20	----------	NA
		BBDCP324-10	JF868695	NA
		BNNR218-15	KX422294	Female
		FIDIP356-11	OK065252	Male
		GMOZH017-21	----------	NA
		GMOZZ012-21	----------	NA
		UAMIC914-13	KU874512	NA
		ZMBN1177-18	----------	Male
***Calliphora mesay* sp. n.**	2	**KEIB029-24**	**PQ593985**	Male
		KEIB030-24	**PQ593986**	Female
Calliphora nigribarbis	11	**KEIB015-24**	**PQ428994**	Male
		----------	MK893470.1	NA
		----------	MT017724	NA
		GBDP30391-19	KY001863	NA
		GBMIN53152-17	KY001864	NA
		GBMNE26664-21	MW085106	NA
		GBMNE26665-21	MW085105	NA
		GBMNF24042-22	LC684778	NA
		GBMNF24043-22	LC684779	NA
		GBMNF24044-22	LC684780	NA
		GBMNE57434-22	OK560152	NA

Calliphora rohdendorfi	2	**KEIB041-24**	**PQ593995**	Male
		KEIB017-24	**PQ593980**	Female
Calliphora sinensis	1	----------	MT017707.1	NA
Calliphora sp. n. 3	1	**KEIB001-24**	**PQ593971**	Female
Calliphora splendens	4	**KEIB018-24**	**PQ428995**	Male
		----------	PP212795.1	NA
		----------	PP212794.1	NA
		----------	PP212793.1	NA
Calliphora stelviana	10	**KEIB019-24**	**PQ428996**	Male
		POLCO6961-20	----------	NA
		AMCAU005-21	----------	NA
		AMCAU361-21	----------	NA
		BNNR221-15	KX422289	Male
		FCHAR6024-19	----------	NA
		FCHAR8618-19	----------	NA
		FIDIP3133-12	MZ623789	Male
		FIDIP4432-16	MZ628261	NA
		FIDIP4433-16	MZ627976	NA
Calliphora subalpina	10	**KEIB048-24**	**PQ594009**	Male
		KEIB020-24	**PQ593981**	Male
		KEIB021-24	**PQ593982**	Male
		KEIB022-24	**PQ428997**	Male
		FIDIP4447-16	MZ623961	NA
		FIDIP461-11	MZ610695	Female
		FIDIP483-11	MZ624790	Male
		FIDIP484-11	MZ622758	Male
		FIDIP517-11	MZ628897	Female
		ZMBN1180-18	----------	Male
***Calliphora teraramma* sp. n.**	3	**KEIB028-24**	**PQ593984**	Male
		KEIB031-24	**PQ593987**	Male
		KEIB032-24	**PQ593988**	Female
Calliphora terraenovae	11	**KEIB023-24**	**PQ594006**	Male
		UAMU096-14	AQM38105.1	NA
		UAMU104-14	AQM38101.1	NA
		UAMU116-14	AQM38096.1	NA
		UAMU175-14	AQM38092.1	NA
		UAMU123-14	AQM38085.1	NA
		UAMU163-14	AQM38083.1	NA
		UAMU153-14	AQM38078.1	NA
		UAMU140-14	AQM38072.1	NA
		UAMU139-14	AQM38071.1	NA
		BNNR217-15	ARO47968.1	Male
Calliphora uralensis	9	**KEIB038-24**	**PQ593993**	Male
		KEIB024-24	**PQ594004**	Male
		----------	MT017722.1	NA
		DRYAS156-14	----------	NA
		FIDIP4434-16	MZ625547	NA

		FIDIP4435-16	MZ627792	NA
		GBMND46119-21	MW564253	NA
		GBMND46120-21	MW565994	NA
		GBMND46121-21	MW566104	NA
Calliphora vicina	13	**KEIB025-24**	**PQ593983**	Male
		KEIB033-24	**PQ593989**	Male
		----------	OY288238	Female
		GBDP15168-14	KF225196	NA
		GBDP16050-15	KJ394580	NA
		GBDP16070-15	KJ394600	NA
		GBDP16112-15	KJ394642	NA
		GBDP7916-09	EU880188	NA
		NICC001-13	KF918981	NA
		NICC004-13	KF918984	NA
		GBDP29017-19	JQ307762	NA
		GBDP7913-09	EU880191	NA
		NICC010-13	KF918990	Male
Calliphora vomitoria	12	**KEIB034-24**	**PQ593990**	Female
		----------	MT584151	NA
		NICC021-13	KF919001	Male
		NICC018-13	KF918998	Male
		NICC028-13	KF919008	Female
		GBMNA9797-19	KT444440	NA
		NICC016-13	KF918996	Male
		GBDP30739-19	MG969490	NA
		NICC026-13	KF919006	Female
		GBDP30737-19	MG969488	NA
		GBDP30738-19	MG969489	NA
		NICC027-13	KF919007	Female
Calliphora zaidamensis	1	**KEIB027-24**	**PQ594005**	Female
Cynomya mortuorum	12	**KEIB068-24**	**PQ664986**	Male
		----------	MT628574	NA
		UAMIC4913-24	----------	NA
		UAMIC4909-24	----------	NA
		UAMIC922-13	KU874772	NA
		FIDIP3129-12	MZ626608	Female
		FIDIP406-11	MZ610745	Male
		GBMIN30488-13	FR719159	NA
		GBMIN53177-17	KY031812	NA
		GRAFW1225-12	KU374728	NA
		NICC038-13	KF919018	NA
		ZERO023-11	KU373567	Male

Table S3. Cytochrome oxidase subunit I (COI), interspecific genetic pairwise distance matrix. Species in bold letters are two newly described species.

Species	*Calliphora calliphoroides*	*Calliphora vomitoria*	*Calliphora uralensis*	*Calliphora rohdendorfi*	*Calliphora croceipalpis*	*Calliphora alaskensis*	*Calliphora grahami*	*Calliphora himalayana*	*Calliphora vicina*	***Calliphora teraramma***	***Calliphora mesay***	*Calliphora subalpina*	*Calliphora loewi*	*Calliphora terraenovae*	*Calliphora sp n 3*	*Calliphora genarum*	*Calliphora nigribarbis*	*Calliphora splendens*	*Calliphora stelviana*	*Calliphora zaidamensis*	*Calliphora chinghaiensis*	*Calliphora sinensis*	*Calliphora latifrons*	*Cynomya mortuorum*
Calliphora calliphoroides	0	5.61	5.8	4.11	7.25	4.1	7.94	7.11	3.29	5.94	4.11	4.44	4.29	5.3	7.18	5.48	4.91	7.17	5.51	5.8	7.94	4.45	7.27	6.28
Calliphora vomitoria	5.61	0	2.01	4.93	7.16	4.43	6.17	4.99	4.42	5.26	4.94	4.76	4.76	3.63	6.29	4.14	2.49	6.8	4.48	2.01	6.17	4.77	5.95	5.96
Calliphora uralensis	5.8	2.01	0	4.96	6.32	4.45	6.01	5.14	4.45	5.96	4.94	4.78	4.78	3.65	5.97	5.15	2.34	7.01	5.15	0	6.01	4.79	5.8	5.96
Calliphora rohdendorfi	4.11	4.93	4.96	0	6.33	1.86	8	6	1.7	6.11	1.86	2.18	2.18	4.31	4.97	4.46	3.61	3.97	4.81	4.96	8	2.18	5.84	6.13
Calliphora croceipalpis	7.25	7.16	6.32	6.33	0	6.13	8.06	6.71	5.84	8.99	5.98	6.33	6.69	5.79	8.51	6.69	6.11	7.7	6.32	6.32	8.06	6.53	6.51	8.85
Calliphora alaskensis	4.1	4.43	4.45	1.86	6.13	0	6.88	5.66	1.54	5.95	1.7	1.23	1.23	4.13	4.98	4.82	3.45	3.79	4.82	4.45	6.88	1.23	5.12	5.79
Calliphora grahami	7.94	6.17	6.01	8	8.06	6.88	0	6.15	7.26	7.93	7.78	6.86	7.23	7.11	7.58	6.63	6.72	10.1	6.77	6.01	0	7.26	7.83	9.26
Calliphora himalayana	7.11	4.99	5.14	6	6.71	5.66	6.15	0	5.66	7.09	6.2	5.64	6	5.32	7.79	5.9	5.29	8.81	5.53	5.14	6.15	6.02	7.44	8.21
Calliphora vicina	3.29	4.42	4.45	1.7	5.84	1.54	7.26	5.66	0	5.42	1.08	2.02	2.02	4.12	5.3	4.13	3.44	4.12	4.15	4.45	7.26	1.87	5.14	6.51
Calliphora teraramma	5.94	5.26	5.96	6.11	8.99	5.95	7.93	7.09	5.42	0	6.45	6.29	5.79	5.86	6.43	5.61	5.79	8.38	6.37	5.96	7.93	5.95	6.89	8.18
Calliphora mesay	4.11	4.94	4.94	1.86	5.98	1.7	7.78	6.2	1.08	6.45	0	2.18	2.17	4.96	5.81	4.99	4.26	3.96	5.35	4.94	7.78	2.02	5.64	5.96
Calliphora subalpina	4.44	4.76	4.78	2.18	6.33	1.23	6.86	5.64	2.02	6.29	2.18	0	0.61	4.12	5.65	5.16	4.1	4.63	5.51	4.78	6.86	0.61	5.84	6.49
Calliphora loewi	4.29	4.76	4.78	2.18	6.69	1.23	7.23	6	2.02	5.79	2.17	0.61	0	4.31	5.29	5.15	4.1	4.62	5.51	4.78	7.23	0.3	5.51	6.34
Calliphora terraenovae	5.3	3.63	3.65	4.31	5.79	4.13	7.11	5.32	4.12	5.86	4.96	4.12	4.31	0	6.4	4.66	2.18	7.37	4.66	3.65	7.11	4.47	5.16	6.55
Calliphora sp n 3	7.18	6.29	5.97	4.97	8.51	4.98	7.58	7.79	5.3	6.43	5.81	5.65	5.29	6.4	0	6.55	5.97	7.73	6.92	5.97	7.58	5.31	7.04	8.06
Calliphora genarum	5.48	4.14	5.15	4.46	6.69	4.82	6.63	5.9	4.13	5.61	4.99	5.16	5.15	4.66	6.55	0	4.31	5.96	3.15	5.15	6.63	5.17	5.52	7.28
Calliphora nigribarbis	4.91	2.49	2.34	3.61	6.11	3.45	6.72	5.29	3.44	5.79	4.26	4.1	4.1	2.18	5.97	4.31	0	6.3	4.63	2.34	6.72	4.11	4.93	6.11
Calliphora splendens	7.17	6.8	7.01	3.97	7.7	3.79	10.1	8.81	4.12	8.38	3.96	4.63	4.62	7.37	7.73	5.96	6.3	0	7.36	7.01	10.1	4.63	7.34	7.35
Calliphora stelviana	5.51	4.48	5.15	4.81	6.32	4.82	6.77	5.53	4.15	6.37	5.35	5.51	5.51	4.66	6.92	3.15	4.63	7.36	0	5.15	6.77	5.53	6.21	6.85
Calliphora zaidamensis	5.8	2.01	0	4.96	6.32	4.45	6.01	5.14	4.45	5.96	4.94	4.78	4.78	3.65	5.97	5.15	2.34	7.01	5.15	0	6.01	4.79	5.8	5.96
Calliphora chinghaiensis	7.94	6.17	6.01	8	8.06	6.88	0	6.15	7.26	7.93	7.78	6.86	7.23	7.11	7.58	6.63	6.72	10.1	6.77	6.01	0	7.26	7.83	9.26
Calliphora sinensis	4.45	4.77	4.79	2.18	6.53	1.23	7.26	6.02	1.87	5.95	2.02	0.61	0.3	4.47	5.31	5.17	4.11	4.63	5.53	4.79	7.26	0	5.49	6.5
Calliphora latifrons	7.27	5.95	5.8	5.84	6.51	5.12	7.83	7.44	5.14	6.89	5.64	5.84	5.51	5.16	7.04	5.52	4.93	7.34	6.21	5.8	7.83	5.49	0	7.09
Cynomya mortuorum	6.28	5.96	5.96	6.13	8.85	5.79	9.26	8.21	6.51	8.18	5.96	6.49	6.34	6.55	8.06	7.28	6.11	7.35	6.85	5.96	9.26	6.5	7.09	0

Table S4. Summary of species delimitation analysis by Automatic Barcode Gap Discovery (ABGD) and Assemble Species by Automatic Partitioning (ASAP), conducted on Spart Explorer (https://spartexplorer.mnhn.fr/).

Number of morpho species		Number of delimited species	ASAP-score	P-val (rank)	W (rank)	Threshold distance
24	ASAP	22	3	1.263941e-02 (3)	0.000051 (3)	0.009878
		13	4.5	1.085083e-03 (1)	0.000017 (8)	0.025836
		25	5.5	6.706587e-01 (10)	0.000061 (1)	0.006839
		30	6	4.870259e-01 (8)	0.000049 (4)	0.005319
		23	7.5	7.924152e-01 (13)	0.000060 (2)	0.008359
	ABGD		Number of Subsets			
		P (prior)	Initial partitions		Recursive partitions	
		0.001	22		25	
		0.001274	22		25	

Note: ASAP-score – A summary statistic incorporating both p-val and W used to rank species partitions.

p-val – The probability that each partition in the analysis contains a single species

W – The relative barcode gap width between the proposed partitioning scheme and the previous scheme.

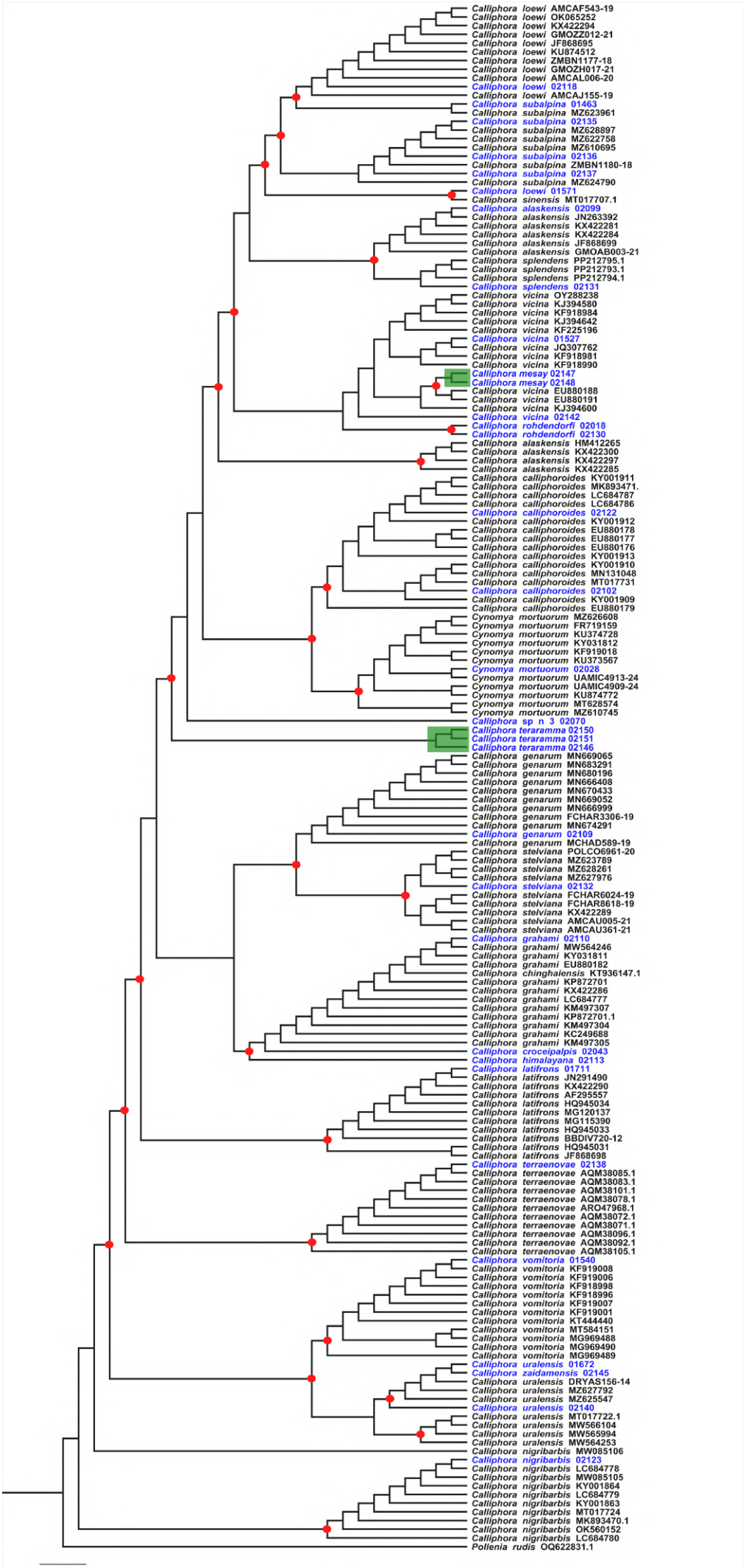
Threshold distance – The midpoint between the current distance that resulted in merging of partitions and the midpoint distance used in the previous partitioning scheme.

p-val and W values are also ranked in their respective columns as indicated in parentheses

Supplementary Figures:

Fig. S1. Maximum likelihood tree for *Calliphora* and *Cynomya* based on mitochondrial cytochrome 1 (COI), using IQ-TREE under BIC-based model selection. The dataset includes COI barcodes of 178 taxa, including Polleniidae as an outgroup. Red circles indicate branches with >80% bootstrap support. Specimens barcoded in this study are highlighted in blue. The clades with two newly described species are marked in green. For clarity, voucher codes or Genbank accession numbers are included along with the species with more than one specimen.

Fig. S2. Species delimitation results for COI sequences (177 individuals, 24 nominal species) analyzed using two distance-based methods (ABGD, ASAP) in Spart Explorer. Colored bars represent species groups delimited by each method, with individual counts shown numerically. The ASAP-score (lower value) and inferred species number (upper value) are displayed above the bars. The cladogram provides phylogenetic support for the delimitation patterns.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Calliphora loewi OK065252
Calliphora loewi KX422294
Calliphora loewi GMOZZ012-21
Calliphora loewi JF868695
Calliphora loewi KU874512
Calliphora loewi ZMBN1177-18
Calliphora loewi GMOZH017-21
Calliphora loewi AMCAL006-20
Calliphora loewi 02118
Calliphora loewi AMCAJ155-19
Calliphora subalpina 01463
Calliphora subalpina MZ623961
Calliphora subalpina 02135
Calliphora subalpina MZ628897
Calliphora subalpina MZ622758
Calliphora subalpina MZ610695
Calliphora subalpina 02136
Calliphora subalpina ZMBN1180-18
Calliphora subalpina 02137
Calliphora subalpina MZ624790
Calliphora loewi 01571
Calliphora sinensis MT017707.1
Calliphora alaskensis 02099
Calliphora alaskensis JN263392
Calliphora alaskensis KX422281
Calliphora alaskensis KX422284
Calliphora alaskensis JF868699
Calliphora alaskensis GMOAB003-21
Calliphora splendens PP212795.1
Calliphora splendens PP212793.1
Calliphora splendens PP212794.1
Calliphora splendens 02131
Calliphora vicina OY288238
Calliphora vicina KJ394580
Calliphora vicina KF918984
Calliphora vicina KJ394642
Calliphora vicina KF225196
Calliphora vicina 01527
Calliphora vicina JQ307762
Calliphora vicina KF918981
Calliphora vicina KF918990
Calliphora mesay 02147
Calliphora mesay 02148
Calliphora vicina EU880188
Calliphora vicina EU880191
Calliphora vicina KJ394600
Calliphora vicina 02142
Calliphora rohdendorfi 02018
Calliphora rohdendorfi 02130
Calliphora alaskensis HM412265
Calliphora alaskensis KX422300
Calliphora alaskensis KX422297
Calliphora alaskensis KX422285
Calliphora calliphoroides KY001911
Calliphora calliphoroides MK893471.
Calliphora calliphoroides LC684787
Calliphora calliphoroides LC684786
Calliphora calliphoroides 02122
Calliphora calliphoroides KY001912
Calliphora calliphoroides EU880178
Calliphora calliphoroides EU880177
Calliphora calliphoroides EU880176
Calliphora calliphoroides KY001913
Calliphora calliphoroides KY001910
Calliphora calliphoroides MN131048
Calliphora calliphoroides MT017731
Calliphora calliphoroides 02102
Calliphora calliphoroides KY001909
Calliphora calliphoroides EU880179
Cynomya mortuorum MZ626608
Cynomya mortuorum FR719159
Cynomya mortuorum KU374728
Cynomya mortuorum KY031812
Cynomya mortuorum KF919018
Cynomya mortuorum KU373567
Cynomya mortuorum 02028
Cynomya mortuorum UAMIC4913-24
Cynomya mortuorum UAMIC4909-24
Cynomya mortuorum KU874772
Cynomya mortuorum MT628574
Cynomya mortuorum MZ610745
Calliphora sp n 3 02070
Calliphora teraramma 02150
Calliphora teraramma 02151
Calliphora teraramma 02146
Calliphora genarum MN669065
Calliphora genarum MN683291
Calliphora genarum MN680196
Calliphora genarum MN666408
Calliphora genarum MN670433
Calliphora genarum MN669052
Calliphora genarum MN666999
Calliphora genarum FCHAR3306-19
Calliphora genarum MN674291
Calliphora genarum 02109
Calliphora genarum MCHAD589-19
Calliphora stelviana POLCO6961-20
Calliphora stelviana MZ623789
Calliphora stelviana MZ628261
Calliphora stelviana MZ627976
Calliphora stelviana 02132
Calliphora stelviana FCHAR6024-19
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Fig. S1

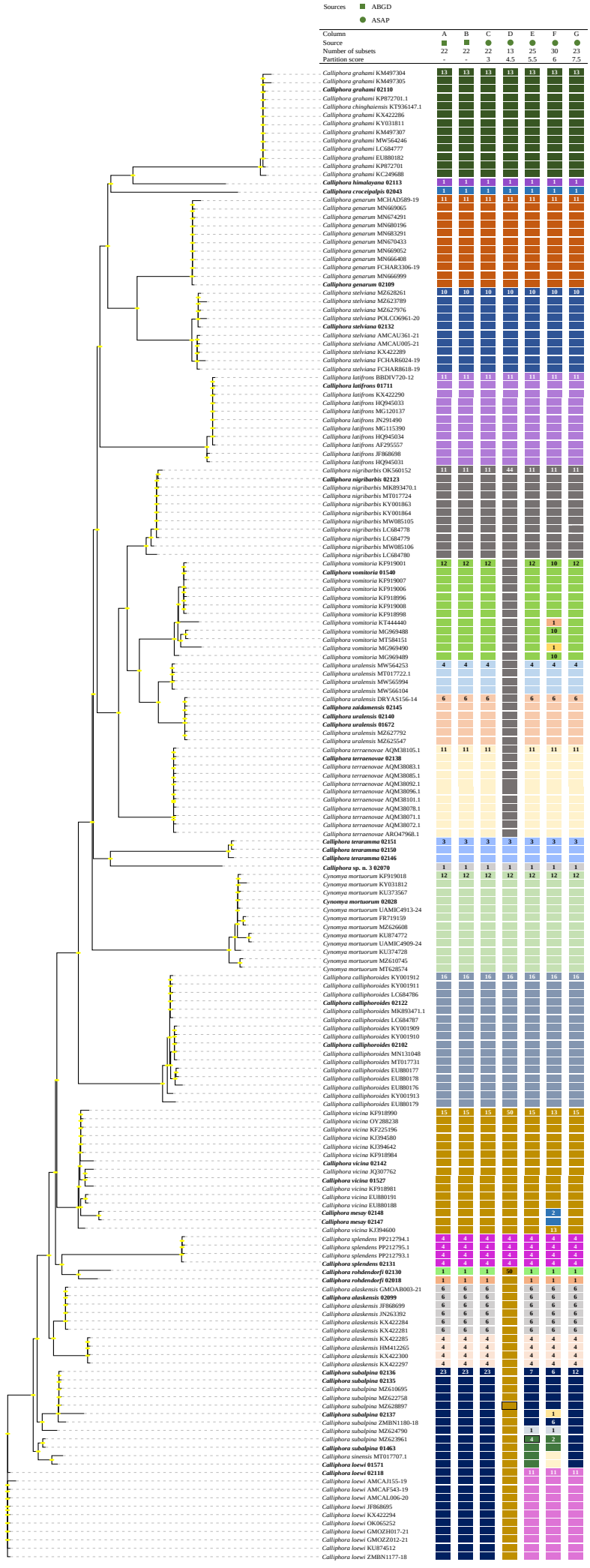
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Fig. S2

ARTICLE 4

Mitogenomic Phylogeny of the Green Bottles (Luciliinae: Calliphoridae: Oestroidea) Reveals Refined Systematics, Biogeographic Patterns, Mid-Miocene Divergence and Repeated Evolution of Parasitism

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Abstract

The blowfly subfamily Luciliinae (Calliphoridae) represents a remarkable case of ecological diversification, with lineages exhibiting saprophagy, facultative parasitism, and obligate myiasis—a spectrum of trophic habits with profound forensic, veterinary, and evolutionary implications. Despite their significance, their origin and evolutionary trajectories remain poorly resolved, hindered by sparse molecular data and conflicting phylogenetic hypotheses. Here, we present the most comprehensive mitogenomic dataset of Luciliinae to date, leveraging 36 newly assembled mitochondrial genomes, including previously unsampled taxa such as *Blepharicnema splendens* Macquart and the Afrotropic endemic *Lucilia infernalis* Villeneuve. This data set is used to investigate green bottle evolutionary history by reconstructing phylogenetic relationships, estimate divergence times, and trace ancestral traits. Our analyses reveal 1) conserved mitogenome structure, 2) strong support for the paraphyly of *Lucilia* Robineau-Desvoidy, 3) divergence dating indicating the presence of early Luciliinae lineages, including *L. infernalis*, in the Afrotropics (~15–20 Ma), and 4) ancestral-state reconstructions demonstrating repeated transitions to parasitism, with saprophagous habits as ancestral state. By integrating mitogenomics, phylogenetics, divergence

dating, trait evolution and historical biogeography, this study provides a unified framework for understanding Luciliinae evolution and has implied importance through refining species boundaries in forensic applications, predicting range shifts under climate change, and developing targeted pest management strategies that account for the evolutionary lability of parasitic traits in this ecologically and economically critical group.

Keywords:
Luciliinae; Mitogenomics; Divergence; Molecular dating; Ancestral state reconstruction; Phylogenetics; Biogeography; Blowfly; Life History; Evolution

1. Introduction

The subfamily Luciliinae (Calliphoridae) comprises five genera–*Blepharicnema* Macquart; *Dyscritomyia* Grimshaw, *Hemipyrellia* Townsend, *Hypopygiopsis* Townsend, and *Lucilia* Robineau-Desvoidy (Whitworth 2014). While several members contribute to decomposition of vertebrate and invertebrate carrion and are considered forensically important, others exhibit diverse parasitic habits and host preferences that enable them to cause myiasis – the infestation of live vertebrate tissues by fly larvae. These breeding habits range from facultative necrophagous myiasis, as seen in *Lucilia sericata* Meigen, which is typically saprophagous but can develop in living tissue, to obligate carnivorous myiasis such as in *Lucilia bufonivora* Moniez, which requires a live amphibian host to complete development (Zumpt 1965; Stevens and Wall 1997; Fremdt et al. 2012). The evolution of facultative parasitism in Luciliinae has been linked to the presence of humans and the domestication of animals, with the shift attributed more to changes in host traits than to major evolutionary change in the flies themselves. Although largely inferential, this hypothesis—particularly in the context of sheep-infesting blowflies—is supported by the suggestions that artificial selection for traits such as dense wool in domestic sheep (*Ovis aries* Linnaeus) has increased their susceptibility to blowfly infestation compared with primitive breeds (Zumpt 1965; Stevens and Wall 1997; Stevens et al. 2006; Stevens and Wallman 2006). Similar to *L. bufonivora*, a highly specialized form of obligate parasitism targeting amphibians—particularly toads—has been reported in *Lucilia elongata* Shannon and *Lucilia silvarum* Meigen (Bolek and Coggins 2002; Bolek and Janovy 2004; Tantawi and Whitworth 2014). However, the feeding habits of *L. silvarum* remains debated. While it has

occasionally been implicated in amphibian myiasis, particularly in North American records, these cases may in part reflect historical misidentifications with *L. bufonivora* due to limitations in earlier morphological keys (Arias-Robledo et al. 2019). Most ecological studies describe *L. silvarum* as a predominantly saprophagous species, developing in decaying animal remains and acting as a primary colonizer of mammalian carrion, including domestic cats (Hanski 1987; Fremdt et al. 2012; Bagsby and Hans 2024), with parasitism considered rare or incidental. Members of the Hawaiian Island endemic genus *Dyscritomyia* are associated with carrion breeding and parasitism of snails (Hardy 1981; Stevens et al. 2002), while species of *Hemipyrellia* are primarily facultative saprophages, and *Hypopygiopsis* includes both facultative parasites and saprophages (Williams et al. 2016). The monotypic *Blepharicnema*—a large Neotropical blowfly (12–14 mm) endemic to the Andes—is strongly attracted to fish bait (Amat and Wolff 2007), although its larval development in vertebrate carrion has not been documented. The ecological diversity and broad geographic spread of Luciliinae highlight their adaptive radiation and complex evolutionary history, which is thought to involve independent transitions from saprophagy to parasitism, potentially driven by biogeographic and climatic factors (Hall and Wall 1995).

Notably, *L. sericata*, *L. caesar* Linnaeus and *Lucilia cuprina* Wiedemann are major myiasis agents with significant forensic and veterinary relevance due to their impact on humans and livestock (Greenberg 1984; Rognes 1998). *Lucilia sericata* and *L. cuprina*, widespread across sheep-rearing regions like Australia, New Zealand, South Africa, and the UK, cause substantial economic losses despite existing control measures (Heath and Bishop 2006; Bambaradeniya et al. 2023). These impacts may intensify under climate change scenarios, which could shift species distributions, alter host interactions and increase fly abundance (Pinto et al. 2021). Complicating these projections further are anthropogenic factors such as global livestock trade and air travel that have contributed to the cosmopolitan spread of these species (Dymock and Forgie 1993; Brett et al. 2021). A recent comparative genomic analysis revealing key subspecies-specific genetic underpinning of divergent ecological roles like parasitism and insecticide resistance in *L. c. cuprina* Wiedemann and *L. c. dorsalis* Robineau-Desvoidy further highlights the importance of evolutionary dynamics in developing targeted pest control strategies, such as genetic biocontrol through engineered fly strains (Kapoor et al. 2025).

Given the complex ecological and biogeographic context of Luciliinae, molecular phylogenetic approaches are essential for uncovering the evolutionary mechanisms underlying parasitic adaptation. Early attempts to explore the evolution of myiasis in blowflies were carried out in morphology- and molecular-based studies (Stevens and Wall 1997; Otranto and Stevens 2002; McDonagh and Stevens 2011; Anstead et al. 2015), but these were methodologically limited, often relying on small taxon sets and lacking formal ancestral state reconstructions or statistical testing. More recent analyses [e.g., (Williams et al. 2016; Arias-Robledo et al. 2019)] improved sampling and integrated additional molecular data, yet still did not address key transitions across all parasitic forms with robust comparative methods. The most comprehensive attempt so far is that of Cardoso et al. (Cardoso et al. 2025), which reconstructed myiasis evolution across blowflies; however, sparse sampling—particularly within Luciliinae—continues to limit resolution of trait transitions. To capture the full spectrum of parasitic adaptations within Luciliinae, denser taxon sampling and the integration of biogeographic context are needed. This would allow finer-scale detection of shifts between larval feeding habits and support more effective, evolution-informed strategies for managing myiasis-causing species.

Despite their ecological and economic relevance—particularly in livestock-associated myiasis and forensic science—the phylogenetic relationships and evolutionary pathways within Luciliinae remain poorly understood. Morphological ambiguities and cryptic speciation continue to obscure species boundaries among closely related or hybridizing taxa such as *Lucilia illustris* Meigen and *L. caesar*, *L. mexicana* Macquart and *L. coeruleiviridis* Macquart, and *L. cuprina* and *L. sericata* (Wallman et al. 2005; Sonet et al. 2012; Debry et al. 2013; Williams and Villet 2013; Williams et al. 2016). Molecular tools have been increasingly used to address these issues, but prior phylogenetic studies have been restricted to economically important species and biased by geographic region, leading to fragmented data and unresolved classification, particularly regarding the monophyly of *Lucilia*—the largest and most studied genus—and the placement of less-studied genera such as *Blepharicnema, Hemipyrellia* and *Dyscritomyia* (Wallman et al. 2005; Harvey et al. 2008; Park et al. 2009; Tourle et al. 2009; McDonagh and Stevens 2011; Sonet et al. 2012; Debry et al. 2013; Williams et al. 2016; Wolff and Kosmann 2016; Cardoso et al. 2025).

Mitochondrial genomes (mitogenomes) have emerged as powerful datasets in insect phylogenetics due to their high-copy number and recoverability from low-quality

or degraded DNA (Cameron 2014; Junqueira et al. 2016; Shang et al. 2022; Johnston et al. 2023). Although the evolutionary inferences based on mitogenome datasets can be influenced by substitution saturation, compositional bias, and accelerated evolutionary rates in some lineages, with introduction of long-branch attraction artifacts, potentially leading to misleading tree topologies and divergence time estimates (Song et al. 2010), these effects can be reduced with appropriate partitioning, recoding, and site-heterogeneous models (Dowton et al. 2009; Song et al. 2010). Moreover, for many rare or historical specimens, mitogenomes remain the most practical—and often the only—genomic resource for broad-scale phylogenetic sampling, enabling robust evolutionary inferences when analyzed with appropriate models and careful taxon selection, even in the absence of complementary nuclear datasets (Dowton et al. 2009; Song et al. 2010; Cameron 2014; Shang et al. 2022). Despite its significance, the application of mitogenomes across Luciliinae has been limited, primarily focused on species identification (Chen et al. 2004; Wallman et al. 2005; Harvey et al. 2008; Park et al. 2009; Nelson et al. 2012; Sonet et al. 2012; Debry et al. 2013). Despite technological advances, comprehensive mitogenomic resources in Luciliinae remain sparse (Parmar et al. 2025). As of August 2025, only 40 complete or near-complete mitogenomes from 11 species (across just two genera) are publicly available (GenBank). Existing mitogenomic datasets remain heavily biased towards *Lucilia* (>97%), with *L. sericata* and *L. cuprina* comprising the majority (>60%) of publicly available sequences (Stevens et al. 2008; Nelson et al. 2012; Junqueira et al. 2016; Shang et al. 2022; Kapoor et al. 2023; Kapoor et al. 2024). Broader evolutionary patterns, including divergence times and transitions in larval life history, have thus remained poorly understood [e.g., (Wallman et al. 2005; Junqueira et al. 2016; Arias-Robledo et al. 2019; Cardoso et al. 2025)].

To resolve these gaps, a more comprehensive phylogenetic and evolutionary framework is needed, incorporating extensive taxon sampling across the full geographic and ecological diversity of Luciliinae. By expanding mitogenomic coverage and integrating biogeographic data, the evolutionary origins, timing, and transitions of parasitic traits can be more accurately inferred. Moreover, trait evolution can be contextualized within historical dispersal events and regional adaptations, offering a deeper understanding of lineage-specific evolutionary drivers.

To this end, 36 complete or near-complete mitochondrial genomes were assembled from 32 species across four Luciliinae genera using genome skimming. This

approach enabled us to generate the first mitogenomic dataset of several previously unsampled taxa. Our comprehensive dataset enabled us to resolve long-standing taxonomic ambiguities in Luciliinae, estimate an evolutionary timescale for their origin and diversification, and investigate the evolution of parasitic habits across biogeographic regions. By addressing these objectives, a more complete evolutionary narrative of Luciliinae is proposed—one that links parasitic trait evolution with biogeographic history and divergence dating.

2. Material and Methods

2.1 Taxon sampling

Adult Luciliinae specimens were both freshly collected during fieldwork and obtained from preserved museum collections (Fig. S1, Table S1). Digital documentation of specimens was performed at Nicolaus Copernicus University in Toruń (NCU) using an M205 C Leica Stereomicroscope with an integrated high-resolution Leica DFC495 digital camera and Leica Application Suite 4.4.0 software. DNA was extracted from 1–2 hind legs of pinned specimens or thoracic tissue from ethanol-preserved specimens. All voucher specimens are housed at the Department of Ecology and Biogeography, Nicolaus Copernicus University in Toruń, Poland.

In total, 36 complete or near-complete mitogenomes were assembled, representing 32 Luciliinae species across four genera, following the classification of Knut Rognes (Rognes K. 1991) and Liping Yan et al. (Yan, Pape, et al. 2021). Geographical origins of specimens were included as tip labels across all analyses. Two datasets were generated for downstream analyses: 1) For phylogenetic inferences, we included additional mitogenomes from NCBI, resulting in a final dataset of 64 Luciliinae specimens representing 35 species. Fourteen additional species from the remaining calliphorid subfamilies (12 genera) and the monotypic Ulurumyiidae (used as outgroup) were also added. 2) For divergence time estimation and ancestral state reconstruction, duplicate species with similar biogeographic origins were removed from the first dataset, yielding 50 Luciliinae specimens (representing 35 species), while retaining all other calliphorid and outgroup taxa. In instances where a species was sampled from geographically distinct regions multiple representatives were retained to account for intraspecific divergence and reconstruct potential dispersal events. This strategy allows for improved estimation of dispersal timing and direction and enables analysis of whether parasitic traits evolved before or after geographic dispersal.

2.2 Mitochondrial genome sequencing, assembly and annotation

Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA), and stored at -80 ºC for downstream analyses. DNA concentration was measured by Qubit 3.0 fluorometer using a dsDNA High Sensitivity Assay Kit (Life Technologies, Inc., Carlsbad, CA, U.S.A.). The quality and integrity of DNA extracts were assessed via gel electrophoresis on 0.5% agarose gels (Biotium, Darmstadt, Germany). Genomic libraries were constructed following a genome skimming protocol (Trevisan et al. 2019), as described in Johnston et al. (Johnston et al. 2023), with the modifications outlined below. Input DNA was mechanically sheared using a Covaris M220 Focused-ultrasonicator (Covaris, Brighton, UK). Two pooled NEBNext Ultra II libraries (insert size: 200 bp) were built and indexed using NEBNext Multiplex Oligos for Illumina (New England BioLabs Inc., Ipswich, MA, U.S.A.). Library size distribution and quality were assessed with an Agilent 2100 Bioanalyser using a high-sensitivity DNA chip (Agilent Technologies, Inc., Santa Clara, CA, U.S.A.). Pippin Prep (Sage Science, Beverly, MA, USA) was used to select fragments between 170–400 bp and eliminate adapter dimers. Libraries were sequenced on an Illumina NovaSeq 6000 platform (2 × 100 bp paired-end) at Macrogen Europe (Amsterdam, The Netherlands), yielding ~1 Gb of clean reads per sample after trimming with Trimmomatic (Bolger et al. 2014).

Mitogenomes were primarily assembled using MitoZ v3.6 (Meng et al. 2019) with the MegaHit v1.2.9 assembler (D. Li et al. 2015), successfully recovering >80% of the genomes. For the remaining assemblies, we applied three additional pipelines using MitoFinder v1.4.2 (Allio et al. 2020), as implemented recently with calyptrates (Johnston et al. 2023): (1) with metaSPADES v4.2.0 (Nurk et al. 2017), (2) with MegaHit v1.2.9, and (3) with IDBA-UD v1.1.3 (Peng et al. 2012), using *Lucilia cuprina* [GenBank: JX913744, (Nelson et al. 2012)] as reference. Combining all methods, we successfully assembled mitogenomes for our entire dataset. Assembly quality and completeness were assessed against published mitogenomes from NCBI.

Gene annotation was conducted using MiTFi v0.1 (Jühling et al. 2012) in MitoZ and ARWEN v1.2.3 (Laslett and Canbäck 2008) in MitoFinder for tRNAs, with tRNA secondary structures predicted using tRNAscan-SE 2.0 Search Server (https://trna.ucsc.edu/tRNAscan-SE/) (Chan and Lowe 2019). The protein coding genes (PCGs) and rRNAs were identified using tblastn (Gertz et al. 2006) and MITOS2 (Bernt et al. 2013), respectively. Tandem Repeats Finder v 4.10.0 (Benson 1999) was used to

detect the A+T-rich control region. Partial or missing genes were annotated using MITOS2 within the Galaxy online interface (https://usegalaxy.eu/) (Afgan et al. 2018). MITOS2 was also used to annotate four mitogenome assemblies of Calliphoridae extracted from National Centre for Biotechnology Information (NCBI). All newly assembled sequences were submitted to GenBank (Table S1).

2.3 Maximum likelihood and bayesian phylogeny

Each of the 13 mitochondrial PCGs was aligned individually using MAFFT v7.490 with the L-INSI algorithm (Katoh and Standley 2013). Poorly aligned taxa and outliers were removed and then each PCG alignment was concatenated into an 11,205 bp supermatrix using SequenceMatrix v1.8.2 (Vaidya et al. 2011). The dataset was then partitioned by gene and codon positions, and phylogenetic inference was conducted using a Maximum likelihood (ML) framework in IQ-Tree v2.1.4-beta (Minh et al. 2020). ModelFinder (Kalyaanamoorthy et al. 2017) within IQ-Tree was used to determine the best-fit evolutionary models and partitioning schemes based on the Bayesian Information Criterion (BIC) (Table S2) (Chernomor et al. 2016). Node support for the majority-rule consensus ML tree was estimated with 10,000 replicates each for ultrafast bootstrap (Hoang et al. 2018) and SH-Like approximate likelihood ratio tests (Guindon et al. 2010).

The final dataset was also analyzed using a Bayesian Inference (BI) approach in ExaBayes v.1.5 (Aberer et al. 2014), running 16 independent MCMC (Markov chain Monte Carlo) chains for 1.5 million generations, sampling every 1,000 generations and discarding the first 25% as burn-in. All priors were kept as default values. Effective Sample Size (ESS > 200) and the Potential Scale Reduction Factor (PSRF < 1.1) were assessed for statistical congruence using 'postProcParam', while topological congruence was evaluated via Average Standard Deviation of Split Frequencies (ASDSF < 0.05). The extended majority-rule consensus tree was generated from sixteen independent runs using the 'consense' function.

To assess the impact of compositional bias and rate heterogeneity on phylogenetic inference, we repeated ML and BI analyses excluding the third codon position, a common approach to reduce substitution saturation (Dowton et al. 2009; Song et al. 2010; Yang et al. 2018).

To test for *Lucilia* monophyly, we conducted a constrained ML analysis enforcing *Lucilia* as monophyletic, using the same model parameters as the unconstrained tree. Topology tests—Approximately Unbiased (AU) (Shimodaira,

2002), Shimodaira-Hasegawa (SH) (Shimodaira and Hasegawa 1999), and Kishino-Hasegawa (KH) (Kishino and Hasegawa 1989) were performed in IQ-TREE using 10,000 RELL bootstrap replicates (-au option) (Kishino et al. 1990) to assess whether the constrained topology differed significantly from the unconstrained topology, thereby quantifying the impact of enforcing monophyly on overall fit to the data.

2.4 Bayesian time-calibrated phylogeny

Divergence time estimation was conducted using a Bayesian MCMC approach implemented in BEAST v 2.7.7 (Bouckaert et al. 2019). Rate variation across lineages was modeled using an uncorrelated relaxed clock log-normal (Drummond et al. 2006), using normal distribution with a Yule (pure birth) speciation model set as the tree prior. Based on previous estimates of divergence times in Oestroidea (Junqueira et al. 2016), three calibration points were applied: the radiations of Oestroidea, the origin of Calliphoridae and the diversification of Calliphorinae + Luciliinae clade (Table S3). Posterior parameter estimates were obtained from two independent MCMC analyses, sampling every 1000 steps over 100 million generations. A 25% burn-in was applied after evaluating convergence and ensuring effective sample sizes (ESS $\geq$ 200) using Tracer v1.7.2 (Rambaut et al. 2018). Final results were summarized into a single ultrametric maximum clade credibility tree using TreeAnnotator v2.7.7 (Drummond and Rambaut 2007; Berling et al. 2025), with node ages scaled to reflect the mean posterior estimates.

All the trees were visualized with FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) and edited using Inkscape 1.3.2 (https://inkscape.org).

2.5 Ancestral state reconstruction

To investigate the tempo and mode of larval parasitic trait evolution and directional transitions across Luciliinae, we performed ancestral state reconstructions (ASR) using a maximum likelihood (ML) approach. Marginal ASR was conducted with the *fitMk* and *ace* from the R package phytools 2.0 (Revell 2024) and ape v5.8-1 (Paradis and Schliep 2019), based on an ultrametric time calibrated phylogeny. Each terminal taxon was coded with one of the six discrete character states for larval feeding: saprophagy, facultative parasitism, obligatory parasitism, coprophagy, predation and (facultative parasitism and predation)—based on published data; species with unknown biology were marked as "NA" and were assigned equal probabilities of being any of the six states (Table S4). Given that several carrion-breeding (saprophagous) species,

such as *L. cuprina* and *L. sericata*, are capable of acting as primary or secondary agents of myiasis (Cardoso et al. 2025), we classified them as facultative parasites in this study. The *fitMk* function assumes equal transition probabilities between states and memoryless state changes. The *ace* function estimates marginal ancestral state likelihoods at all nodes.

We further implemented stochastic character mapping (SCM) using the *make.simmap* function in *phytools*, with 1000 MCMC simulations to estimate posterior probabilities of states at nodes and count transitions among character states. Three transition rate models—equal rates (ER), symmetrical (SYM), and all rates different (ARD)—were tested using *ace*, each replicated 10 times with randomized starting values to ensure convergence. The model fit was evaluated based on AICc and AIC weights to determine the relative support for each model (Harmon 2019). To visualize evolutionary transitions and assess ancestral states, a model-averaged marginal ancestral reconstruction was performed using *ancr()*. Feeding mode states were color-coded using a custom palette and visualized on the tree with node pie charts representing marginal probabilities. Ancestral states on the phylogeny were mapped and visualized using the *plotTree* function.

3. Results

3.1 Mitogenomic characteristics

We successfully assembled 36 new mitogenomes for the Luciliinae (Table S1). All assembled mitogenomes exhibited the typical circular, double-stranded structure and gene arrangement found in Diptera, spanning 14–19 kb and containing 37 genes (2 rRNA genes, 13 PCGs, and 22 tRNA genes) and a non-coding, AT-rich control region (Li et al. 2020; Yan, Pape, et al. 2021; Shang et al. 2022). Gene order remained conserved across all taxa, consistent with the ancestral insect arrangement (Cameron 2014).

Luciliinae mitogenomes showed a strong A+T-bias (75.2%–77.7%), with variation among genera—from 75.6% in *Blepharicnema* to 77.5% in *Hemipyrellia* (Table S5). The PCGs reflected similar variation, with A+T content ranging from 74.44% (*Blepharicnema*) to 75.65% (*Hemipyrellia*) (Table S5). Positive AT skew and negative GC skew were observed across all genera, while PCGs showed an inverted pattern (Table S5). Detailed information on base composition, GC skew, and GenBank accession numbers is provided in Tables S1 and S5.

3.2 Phylogenetic analyses

Phylogenetic analyses based on 79 mitogenomes (49 species across all eight calliphorid subfamilies plus an outgroup) yielded well-resolved and largely congruent trees under both Maximum Likelihood (ML) and Bayesian Inference (BI) frameworks (Figs 1–2; Figs S2–S3; Table S1). Calliphoridae and all its subfamilies were consistently recovered as monophyletic. This included the placement of Rhiniinae and Rhinophorinae within Calliphoridae. In contrast, *Eurychaeta palpalis* Robineau-Desvoidy (formerly Helicoboscinae) disrupted the monophyly of Ameniinae.

Under both analytical frameworks, Luciliinae was recovered as monophyletic and sister to Calliphorinae. However, *Lucilia* was rendered paraphyletic across all analyses due to the nested placement of *Blepharicnema*, *Hemipyrellia*, and *Hypopygiopsis* (Figs 1–2, Figs S2–S3). Topology testing strongly rejected the monophyly of *Lucilia*, as evidenced by significant differences between the unconstrained and monophyly-constrained phylogenies (Table S6). The monophyly-constrained tree had a significantly lower log-likelihood (ΔlogL = 0.086341) compared to the unconstrained tree, with the p-value of 4.55e−11 in the approximately unbiased (AU) test. Both the Shimodaira–Hasegawa (SH) and Kishino–Hasegawa (KH) tests also significantly rejected the constrained tree.

The monotypic *Blepharicnema*—included here in a phylogenetic analysis for the first time—clustered consistently within *Lucilia* with strong support (Figs 1–2, Figs S2–S3). Most analyses strongly supported *Hemipyrellia* as a distinct lineage, while species of *Hypopygiopsis* showed inconsistent placements, clustering variably with species of *Lucilia* or *Hemipyrellia* (Figs 1–2, Figs S2–S3).

Species-level relationships within *Lucilia* were generally stable, with minor topological variation under third-codon exclusion (Figs 1–2; Figs S2–S3). *Lucilia sericata* and *L. cuprina* formed a sister pair, although some *L. cuprina* specimens were nested within *L. sericata*. Several other well-supported clades were identified, including the *cuprina–sericata* group and clades containing *L. richardsi* Collin, *L. regalis* Meigen, *L. pilosiventris* Kramer, *L. silvarum*, *L. elongata*, *L. bufonivora*, and *L. thatuna* Shannon (Stevens 2003; McDonagh and Stevens 2011; Debry et al. 2013; Williams et al. 2016; Arias-Robledo et al. 2019; Kapoor et al. 2023). In the majority of our analyses (except in ML12 and BI12), *Lucilia infernalis* Villeneuve was strongly recovered as sister to all other Luciliinae (posterior probability [PP] = 1.0; SH-aLRT = 100.0; ultrafast bootstrap [UFB] = 100.0), in contrast to previous findings (Williams et al.

2016), which placed *L. infernalis* as sister to a *Hemipyrellia* clade, with this combined group nested within *Lucilia* (based on a single marker e.g., COI). Additional species clusters—e.g., (*L. illustris*, *L. caesar*, *L. hainanensis* Fan, *L. porphyrina* Walker, *L. ampullaceal* Villeneuve) + (*L. sinensis* Aubertin, *L. papuensis* Macquart, *L. shenyangensis* Fan, *L. calviceps* Bezzi, *L. bazini* Séguy), and (*L. coeruleiviridis*, *L. eximia* Wiedemann, *L. pulverulenta* Whitworth, *L. albofusca* Whitworth, *L. cluvia* Walker, *L. retroversa* James, *L. fayeae* Whitworth, *L. rica* Shannon)—were consistently recovered and aligned with previous studies, though this analysis includes broader taxon sampling than past efforts (Stevens 2003; Wells et al. 2007; Debry et al. 2013; Kapoor et al. 2023).

Excluding the third codon positions from the Luciliinae dataset resulted in reduced tree resolution and nodal support across analyses, without improving topological accuracy (Figs S2–S3). These outcomes were consistent with patterns observed in previous studies within Diptera, including Calliphoridae (Caravas and Friedrich 2013; Shang et al. 2022).

3.2.1 Geographic structuring and lineage patterns

Phylogenetic analyses revealed a strong biogeographic structure among Luciliinae species. *Lucilia sericata* formed a globally distributed, well-supported clade with minimal mitochondrial divergence across continents (Figs 1–2, Figs S2–S3), indicating a likely invasive haplotype pool (Stevens and Wall 1997; Wallman et al. 2005; McDonagh and Stevens 2011). In contrast, *L. cuprina* lacked clear geographic structure, with Eastern Australian specimens (e.g., Queensland and Wollongong) clustering closer to *L. sericata* than to other *cuprina* lineages of mixed eastern and western origin. Among these, one sequence previously identified as *L. c. cuprina* (MW255538) grouped with *sericata*, while the second specimen was not identified to subspecies level. Moreover, phylogenetic resolution within the *cuprina–sericata* clade was poor (Figs 1–2, Figs S2–S3).

L. porphyrina was paraphyletic, forming two distinct clades: one from Australia and another comprising a Malaysian specimen that clustered with *L. hainanensis* (MW592363) of unknown origin. A well-supported Australasian clade consisting of *L. calviceps*, *L. bazini*, *L. sinensis*, *L. papuensis*, and *L. shenyangensis* was recovered, although the latter three showed paraphyletic relationships. The Holarctic *L. illustris* and Palaearctic *L. caesar* were recovered as distinct lineages with strong support (Figs 1–2, Figs S2–S3). Western Hemisphere taxa including *L. eximia*, *L. rica*, *L.*

coeruleiviridis, *L. pulverulenta*, *L. albofusca*, *L. cluvia*, *L. retroversa* and *L. fayeae* formed a well-supported clade sister to *Blepharicnema splendens* Macquart.

Hemipyrellia formed a well-supported, monophyletic lineage restricted to Southeast Asia and Australia, reinforcing prior reports of its restricted distribution in Eastern-Hemisphere (Wallman et al. 2005; Williams et al. 2016). It was consistently placed within *Lucilia* in all analyses (Figs 1–2, Figs S2–S3). *Hypopygiopsis*, endemic to Australasia (Kurahashi 1977), was split into two well-supported clades (Figs 1–2, Figs S2–S3): one comprising *H. tumrasvini* Kurahashi and *H. fumipennis* Walker (from Thailand and Malaysia, respectively), sister to globally distributed *Lucilia* clade; and the other comprising *H. violacea* Macquart and *H. infumata* Bigot (both from Malaysia), which clustered with *Hemipyrellia* in an exclusively Eastern Hemisphere clade. The two *Hypopygiopsis* clades showed high divergence (p-distance = 7.5%), exceeding that between the Malaysian *Hypopygiopsis* and *Hemipyrellia* (p-distance = 5.3%). Sequence divergence between the two *Hypopygiopsis* clades was high (*p* distance-7.5%). *Lucilia infernalis*, an Afrotropical endemic, was placed as the sister to all other Luciliinae.

3.3 Divergence Time Estimation and Historical Biogeography

Divergence time estimates from two independent MCMCtree runs were congruent, with consistent posterior distributions (Fig. 3). The origin of Oestroidea was estimated at ~37.44 Ma (95% CI: 36.56–38.39), with the core-Calliphoridae diverging around 23.01 Ma (95% CI: 21.13–25.22). Most calliphorid subfamilies diverged between ~20.75 Ma (95% CI: 18.48–23.24) and 15.85 Ma 15.85 Ma (95% CI: 14.63–17.27), with the latter referring to the origin of the sister lineages Luciliinae and Calliphorinae (Fig. 3).

Within Luciliinae, genus-level diversification occurred between ~13.09 Ma and 5.62 Ma (Fig. 3). In contrast, most within-species divergences were recent, generally younger than 4 Ma and concentrated during the Pliocene–Pleistocene. Very shallow divergences (<0.2 Ma) were observed between *Lucilia fayeae* and *L. rica*, *Hypopygiopsis violacea* and *H. infumata*, and *L. cuprina* and *L. sericata*. Most intraspecific divergences fell between 0.1–0.8 Ma, while deeper divergences were observed in *L. papuensis* and *L. porphyrina* (~4.6–5.6 Ma).

Clear geographic structuring was evident across several groups. Neotropical lineages such as *L. eximia*, *L. cluvia*, and *L. retroversa* diversified during the late Miocene (5–9 Ma) (Fig. 3). Palearctic and Nearctic species, including *L. illustris*, *L. caesar*, and *L. sericata*, exhibited much more recent divergences (<2 Ma). The *L.*

cuprina–L. sericata complex formed a shallow clade (~0.18–0.21 Ma), except for a Namibian *L. cuprina* lineage that diverged ~1.87 Ma. Several tropical Eastern-Hemisphere taxa also showed deep divergences. *Lucilia infernalis*, endemic to Africa, represented one of the two lineages produced by the initial split of Luciliinae (~13.09 Ma), while *Hypopygiopsis* species from Southeast Asia diverged between ~6–13 Ma.

3.4 Evolutionary transitions in larval feeding modes

Ancestral-state reconstruction favored the Equal Rates (ER) model (AIC = 173.71; AIC weight = 0.51) and the Symmetrical (SYM) model (AIC = 173.80; AIC weight = 0.49) as nearly equally well-supported explanations of larval feeding mode evolution, whereas the All-Rates-Different (ARD) model (AIC = 228.86; weight ≈ 5.4e–13) was clearly overparameterized and poorly supported. This near-equal support for ER and SYM models suggests that transitions among feeding modes likely occurred at comparable rates across the phylogeny, with little evidence for strongly biased directional changes. Given the minimal difference in fit, the simpler ER framework provides a reasonable basis for interpreting ancestral state shifts.

Analysis of the ancestral-state tree revealed multiple independent shifts in larval feeding modes. In general, early blowfly lineages (around the Oligocene–Miocene transition, ~23 Ma) most likely evolved as either obligate or facultative parasites (mean probabilities: 0.34 and 0.23, respectively) (Figs 3–4). For Luciliinae, the ancestral larval feeding mode for most genera was inferred to favor saprophagy or facultative parasitism (mean probabilities: 0.51 and 0.45, respectively) (Fig. 4).

A shift to obligate parasitism in Luciliinae occurred around 4.2 Ma (95% CI: 3.41–5.12) during the Pliocene (Figs 3–4), and parasitic feeding habits evolved repeatedly between ~9 Ma and 0.2 Ma. The obligate amphibian parasites *Lucilia bufonivora* and *L. elongata* evolved independently, while *L. infernalis*, representing one of the early splits within Luciliinae species (~13.1 Ma), remained saprophagous.

Discussion

4.1 Conserved mitogenome structure and organization revealed within Luciliinae

The conserved structure and gene order observed in Luciliinae mitogenomes align with previously reported patterns across Diptera and other Calliphoridae (Cameron 2014). The high A+T content and skew patterns are characteristic of insect mitogenomes and are likely driven by replication-associated mutation asymmetries (Junqueira et al. 2016; Yan, Xu, et al. 2021; Shang et al. 2022; Kapoor et al. 2024; Pei et al. 2024). The genus-

specific variation in A+T content and skew may reflect lineage-specific mutational biases or selective pressures (Lu et al. 2023).

Although compositional biases such as AT-GC skew have been implicated in rearrangements and strand-specific mutation rates in other metazoans (Perna and Kocher 1995; Hassanin et al. 2005; Wei et al. 2010), no such correlation was evident in Luciliinae. Nevertheless, these biases may indicate deeper evolutionary or metabolic constraints and contribute to mitogenomic stability. While nucleotide composition had minimal influence on phylogenetic resolution in our dataset (see section 3.2), it remains informative for understanding mitogenome evolution and lineage-specific dynamics (Wei et al. 2010; Lu et al. 2023; Ghosh et al. 2024).

4.2 Comprehensive taxon sampling helps to resolve Luciliinae phylogeny and long-contested taxonomic ambiguities

Our expanded mitogenomic dataset substantially improves resolution within Luciliinae and broader Calliphoridae. The consistent recovery of all subfamilies except Ameniinae as monophyletic confirms recent taxonomic revisions and reinforces the inclusion of Rhiniinae and Rhinophorinae within Calliphoridae (Kutty et al. 2010; Yan, Pape, et al. 2021; Cerretti et al. 2024). The monophyletic Luciliinae, forming the sister clade to Calliphorinae in our study, align with previous morphological and molecular evidence (Hennig 1973; Kutty et al. 2010; Marinho et al. 2012; Nelson et al. 2012; Yan, Pape, et al. 2021; Shang et al. 2022; Cerretti et al. 2024).

Crucially, our findings confirm the paraphyly of *Lucilia*, with *Blepharicnema*, *Hemipyrellia*, and *Hypopygiopsis* consistently embedded within it (Figs 1–2, Figs S2–S3), supported by topology tests strongly rejecting its monophyly (Table S6). These results corroborate earlier studies suggesting potential synonymy or reclassification of these genera (Rognes K. 1991; Wells et al. 2007; Park et al. 2009; DeBry et al. 2010; McDonagh and Stevens 2011; Williams et al. 2016; Cardoso et al. 2025). However, we refrain from proposing changes to the classification at this stage pending further morphological and genomic analysis with a larger number of taxa across these genera.

The stable placement of *L. sericata* and *L. cuprina* as closely related but non-monophyletic supports previous findings and highlights ongoing introgression and hybridization between the two species (Wallman et al. 2005; McDonagh and Stevens 2011; Nelson et al. 2012; Sonet et al. 2012; Williams and Villet 2013; Williams et al. 2016; Kapoor et al. 2023). These results underscore the need for taxonomic revision and clearer species delimitation within this complex. Several other *Lucilia* species-

clusters (including *L. illustris, L. caesar, L. sinensis, L. papuensis, L. coeruleiviridis, L. eximia*) received strong nodal support, offering a refined view of intrageneric relationships and expanding on prior efforts with limited Luciliinae representation [e.g., (Stevens 2003; Wells et al. 2007; Debry et al. 2013; Kapoor et al. 2023).

The reduced tree resolution observed upon third codon exclusion indicates that this partitioning strategy compromises phylogenetic signal without substantially mitigating compositional bias or rate heterogeneity. As also noted in prior studies (Caravas and Friedrich 2013; Shang et al. 2022), retaining all three codon positions is optimal for robust mitogenome-based phylogenetic inference in Calliphoridae.

4.2.1 Phylogeographic insights and taxonomic implications

The low mitochondrial variation between globally distributed samples of *L. sericata* supports previous hypotheses of its invasive nature and global dissemination (Williams et al. 2014). In contrast, the lack of clear phylogeographic structure in *L. cuprina* and its paraphyly with respect to *sericata* likely reflects instances of introgression and hybridization. This is consistent with previous nuclear data indicating reciprocal monophyly between the species and reinforces the need for taxonomic reevaluation of this complex (Stevens et al. 2002; Tourle et al. 2009; McDonagh and Stevens 2011; Kapoor et al. 2025). Moreover, the *cuprina–sericata* clade (Figs 1–2, Figs S2–S3) was poorly resolved, and our study therefore does not confirm prior hypotheses of geographic subspecies delineation in Australia—*L. c. cuprina* (eastern) and *L. c. dorsalis* (western) (Gleeson and Heath 1997; Wallman et al. 2005; Harvey et al. 2008; Kapoor et al. 2025).

The paraphyly of *L. porphyrina* and several Australasian *Lucilia* species highlights unresolved taxonomic issues. The repeated recovery of these patterns across studies point to the need for revised classifications supported by data from nuclear genes and broader geographic sampling (Williams et al. 2016; Kapoor et al. 2023). The strong separation of *L. illustris* and *L. caesar* validates their distinct Holarctic and Palaearctic distributions (Debry et al. 2013). Likewise, the well-supported Western Hemisphere clade, including *L. eximia, L. rica, L. coeruleiviridis* and others, aligns with previous observations of regional endemism in the Americas (Wallman et al. 2005).

The placement of *Hemipyrellia* within *Lucilia* in our study challenges its current generic status and supports earlier suggestions of synonymy (Wells et al. 2007; Park et al. 2009; McDonagh and Stevens 2011; Williams et al. 2016). This contradicts previous studies that treated *Hemipyrellia* as a distinct sister lineage to *Lucilia* based on limited

sampling and restricted biogeography (Wallman et al. 2005; Nelson et al. 2012; Singh and Wells 2013). Similarly, the deep divergence between the two *Hypopygiopsis* clades, based on full mitogenomes, counters earlier claims of misidentification based on a single mitochondrial marker, i.e., COI (Williams et al. 2016), and suggests more complex evolutionary histories. However, taxonomic actions such as possible splitting of this genus or synonymizing it with *Lucilia* should await expanded sampling and nuclear genomic data.

Finally, the distinct placement of *L. infernalis* as one of the two basal lineages in our phylogeny, together with its Afrotropical endemism, is consistent with Africa having been part of the ancestral area of Luciliinae, with subsequent diversification of its sister lineage, comprising all other Luciliinae, across other geographic regions. While the observed geographic clustering patterns suggest that historical dispersal and vicariance have likely influenced Luciliinae diversification, a formal biogeographic analysis with broader taxon sampling, particularly including sister lineages (e.g., Calliphorinae), would be required to test these hypotheses rigorously.

4.3 Miocene origins and Pleistocene radiations: geoclimatic drivers of Luciliinae evolution

Our divergence estimates align closely with previous studies [e.g., (Wiegmann et al. 2011; Junqueira et al. 2016)], confirming a late Eocene origin for Oestroidea and early Miocene radiation of core-Calliphoridae [*sensu* (Yan, Pape, et al. 2021)]. While Oestroidea diversification can be linked to the rapid radiation of mammals during the Paleogene period (Stevens 2003; Junqueira et al. 2016), the diversification of core-Calliphoridae is linked to the period of global drying, expansion of grasslands, and rapid radiation of large herbivorous mammals—particularly even-toed ungulates—across Africa and Eurasia (Stebbins 1981; Bredenkamp et al. 2002). The resulting increase in carrion availability likely created new ecological opportunities that facilitated the adaptive radiation of blowflies (Junqueira et al. 2016). Simultaneously, major geophysical changes, such as the closure of seaways between the Mediterranean and Indian Ocean, linking Africa and Eurasia, and the persistence of the Bering land bridge, connecting East Asia and North America, promoted faunal exchange between continents (Zachos et al. 2001).

Compared to previous studies, which sampled Luciliinae sparsely or used limited markers [e.g., (Wallman et al. 2005; Wiegmann et al. 2011; Junqueira et al. 2016; Cerretti et al. 2017; Arias-Robledo et al. 2019)], our expanded sampling enabled

a more refined understanding of the evolutionary history of the subfamily. A Middle Miocene origin of Luciliinae ~15–20 Ma is consistent with earlier work (Fig. 3) [e.g., (Wallman et al. 2005; Wiegmann et al. 2011; Junqueira et al. 2016; Cerretti et al. 2017; Arias-Robledo et al. 2019)]. This timing coincides with the Middle Miocene Climatic Optimum (MMCO), a period of global warmth and biome restructuring—which likely offered new ecological opportunities for diversification into saprophagous and parasitic niches. Environmental shifts—such as uplift of the Andes and Himalayas, and expansion of savannas—likely promoted early allopatric divergence across continents. Our estimates indicate that the initial split within Luciliinae (~13.09 Ma, Middle Miocene) produced two contemporaneous lineages: one represented by *L. infernalis* (endemic to Africa) and the other comprising the remaining Luciliinae, which subsequently diversified across multiple regions, including Asia and the Western Hemisphere, likely facilitated by increased aridification and the expansion of open savannas (Linder 2017). While the placement and distribution of *L. infernalis* are consistent with Africa having been part of the early range of Luciliinae, the present study does not provide sufficient evidence to determine the precise ancestral area, and further formal biogeographic analyses are needed. In particular, most older clades from mid to late Miocene (~6–13 Ma) across the entire subfamily are represented in the Eastern Hemisphere, followed by rapid divergence (over ~5 Ma) in the Western Hemisphere.

Lineage divergences corresponding to currently recognized genera clustered in the Miocene, while most intraspecific divergence—genetic splits within presently recognized species—occurred during the Pliocene and Pleistocene (Fig. 3). These temporal patterns suggest that glacial cycles, habitat fragmentation, and climatic oscillations played major roles in shaping present-day diversity. The shallow splits in *L. cuprina–L. sericata* and other species-pairs point to recent divergence, possible hybridization, ongoing gene flow, and human-mediated anthropogenic spread in the Holocene, especially in livestock-associated synanthropic taxa (Erzinclioglu 1989; Stevens and Wall 1997; Wallman et al. 2005; Stevens et al. 2006; Kapoor et al. 2025).

The deeper divergence of Namibian *L. cuprina* may reflect historical incomplete lineage sorting or introgression within a species complex that is not yet fully genetically or taxonomically resolved or a case of misidentification. Similarly, deep splits between *L. papuensis* and *L. porphyrina* may reflect cryptic speciation or misidentification. The divergence of Neotropical lineages in late Miocene (~5–9 Ma), such as *Lucilia eximia*,

L. cluvia, and *L. retroversa* coincides with Andean uplift and Panama Isthmus closure (~3 Ma) and supports a role for elevational and dispersal barriers in speciation (Bermingham and Martin, 1998). For Palearctic and Nearctic taxa, including *L. illustris*, *L. caesar*, and *L. sericata*, more recent divergences correspond to Pleistocene glaciations and associated expansion-contraction dynamics. While these climatic oscillations likely caused repeated shifts in habitat availability and promoted fragmentation and isolation in glacial refugia, facilitating some speciation between Eurasia and North America (Markgraf et al. 1995), the comparatively modest number of Luciliinae species today suggests that such events alone were not a dominant driver of diversification, and other ecological or historical constraints, such as extinction, limited ecological opportunities, or constraints in dispersal and adaptation may have limited lineage proliferation.

Taken together, these findings highlight the central role of Miocene–Pleistocene geoclimatic changes in generating Luciliinae diversity, likely through region-specific radiations influenced by ecological opportunity and vicariant processes. While such patterns are not unique to Luciliinae and are shared by many other insect clades, they provide important context for understanding the group's present-day distribution and diversification.

4.4 Ancestral State Reconstruction reveals independent origins and homogeneous transitions of larval feeding habits in Luciliinae

Our ancestral-state analysis challenges the traditional view of a stepwise evolution from saprophagy to obligate parasitism via facultative stages (Zumpt 1965; Erzinclioglu 1989; Stevens and Wall 1997; Stevens 2003). Instead, they reveal a more flexible and labile evolutionary trajectory of larval feeding, especially in Luciliinae. The ER model result suggests no strong directional constraint, allowing transitions between any feeding modes.

During the early Miocene (~23–17 Ma), obligate and facultative parasitic habits likely emerged independently in several blowfly lineages. This period coincided with peak gastropod and arthropod diversity (Solórzano Kraemer et al. 2015; Höltke et al. 2016; Grossmann et al. 2023), providing ecological niches for parasitic exploitation. Some obligate invertebrate parasites, such as Ameniinae—macrolarviparous parasites of terrestrial snails—and Rhinophorinae—oviparous parasites of woodlice (oniscid isopods)—along with termite-parasitic Bengaliinae (Rognes K. 1991; Rognes 2011) likely originated in response to such host availability. Although Ameniinae and

Rhinophorinae are coded similarly in our analyses as obligate parasites, their distinct reproductive modes and host associations suggest independent origins from a common ancestor likely exploiting soil-dwelling invertebrates. Associations between some Rhiniinae (e.g., *Stomorhina*) and termites or ants have been observed, but these appear to reflect adult foraging or oviposition behavior rather than confirmed larval feeding habit (parasitism, parasitoidism, predation or scavenging) (Thomas-Cabianca et al. 2023). A few Bengaliinae genera (not included in this study), which likely evolved around the same time, such as *Auchmeromyia* Brauer & Bergenstamn, *Booponus* Aldrich, *Cordylobia* Grünberg, and *Pachychoeromyia* Villeneuve are also obligate mammal parasites (Rognes 2011), coinciding with flourishing mammal diversity following the creation of new habitats in early Miocene (Finarelli and Badgley 2010). Blowflies exploiting live host also appeared in several clades around the Miocene–Pliocene: the snail-parasitic *Melinda* Robineau-Desvoidy lineage (Calliphorinae) and the locust-egg predators *Stomorhina* Rondani (Rhiniinae) arose in the mid to late Miocene.

The expansion of grasslands and woodlands in the early Miocene (~17.69 Ma) coincided with increased diversity and abundance of vertebrate and invertebrate hosts (Strömberg 2011; Steinthorsdottir et al. 2021). This environmental change may have facilitated shifts in larval feeding habits from obligatory parasitism to saprophagy/facultative parasitism, which later became dominant in calliphorid lineages evolved ~17.69 Ma. However, it is important to note that transitions between feeding habits likely differ depending on the ancestral state — for example, shifts from obligate parasitism to facultative parasitism versus from saprophagy to facultative saprophagy may have distinct ecological drivers. Intense competition for ephemeral resources such as carrion can lead to niche partitioning or specialization (Hanski and Kuusela 1977; Hanski 1987). In some cases, high competition may favor specialization to reduce overlap, while in others, facultative habits allowing exploitation of multiple resource types could be advantageous in fluctuating environments [e.g., (Poulin and Morand 2000)]. For instance, the saprophagous ancestor of *Lucilia bufonivora* likely faced intense competition among carrion-breeding flies, potentially driving it to exploit narrower niches, such as infesting injured amphibian hosts and eventually evolving obligate parasitism (Arias-Robledo et al. 2019). Such ecological pressures highlight the complexity of transitions in larval feeding modes, which may not be unidirectional or uniform across taxa. Although our study does not directly test these ecological

mechanisms, the observed timing of diversification and trait evolution is consistent with these ecological hypotheses. Further experimental and ecological studies would be necessary to rigorously test these ideas. The occurrence of saprophagy across calliphorid lineages—larval feeding habits differing from those inferred for the ancestral node (obligate parasitic or facultative parasitic)—arising multiple times as niches shifted, highlights the complex, non-linear evolution of larval feeding habits within the family. In fact, recent phylogenetic modeling based on mitochondrial and nuclear data finds that the root of Calliphoridae was equally likely to be saprophagous or facultatively parasitic (Cardoso et al. 2025), reinforcing the idea of a flexible evolutionary history. However, it should be noted that the present study lacks multiple taxa from other non-luciliine calliphorid subfamilies and therefore, we refrain to infer ancestral state for Calliphoridae as a whole.

Within Luciliinae, saprophagy and facultative parasitism dominate early ancestral states, and obligate parasitism evolved independently at least twice. *L. bufonivora* and *L. elongata* are prime examples, evolving to specialize on amphibians (toad myiasis) (Zumpt 1965; Rognes K. 1991; McDonagh and Stevens 2011; Tantawi and Whitworth 2014) in different geographic and phylogenetic contexts. This pattern matches the prior hypothesis of saprophagous ancestors, suggesting that facultative feeding often bridges to obligate parasitic habits (Cardoso et al. 2025) and that such specialized habits likely arose later, rather than being ancestral traits within Luciliinae (Stevens 2003; Arias-Robledo et al. 2019). Accordingly, we infer that early Luciliinae transitioned from saprophagy toward more flexible feeding modes, whereas the earliest diverging *L. infernalis* retained saprophagy, reinforcing the idea that specialized parasitism arose recently and independently (Figs 3–4). Ecological shifts, involving global warming, restructuring in biomes, such as savanna expansion and mountain uplift during the Middle Miocene Climatic Optimum (~13 Ma) may have created new opportunities for niche expansion that supported both saprophagous and parasitic lifestyles (Böhme 2003; Linder 2017), consistent with observed rapid diversification in Luciliinae, occurring within less than 5 Ma after lineage's origin. This is further supported by the repeated emergence of parasitic life-style between ~9 Ma and the present, particularly during the Pliocene, when cooling climates and increased host availability likely selected for parasitism as a strategy to avoid heavy competition for carrion (Arias-Robledo et al. 2019; Steinthorsdottir et al. 2021).

While host–parasite coevolution is known to drive lineage diversification and speciation in some obligate parasitic oestrid flies (Pape 2006; Stevens et al. 2006), our ancestral state reconstruction indicates that obligate myiasis in *L. bufonivora* and *L. elongata* evolved independently within Luciliinae. These lineages coexist with predominantly facultative parasitic species, suggesting multiple independent adaptations to specialized niches and host availability (Stevens 2003; Arias-Robledo et al. 2019). This finding refutes earlier hypotheses proposing obligate parasitism as a monophyletic trait with a single evolutionary origin within Luciliinae (Arias-Robledo et al. 2019; Cardoso et al. 2025).

The timing of obligate parasitism evolution across Nearctic and Palearctic regions within Luciliinae (~4.2 Ma) suggests a potential link to intercontinental dispersal of amphibian fauna during the Early Pliocene, possibly mediated by Bering Land Bridge (Zachos et al. 2001; J.-T. Li et al. 2015). While the most extant amphibian lineages have appeared to be originated much earlier, in end-Permian mass extinction and in the late Cretaceous [e.g., (Roelants et al. 2007)], climatic shifts during the Pliocene (e.g., increased humidity and habitat fragmentation) may have reshaped amphibian distributions and population dynamics (Barnosky 2005), potentially creating new ecological opportunities for parasites like *L. bufonivora* to exploit hosts in isolated or expanding populations. Alternatively, the fly's specialization may reflect niche partitioning within pre-existing amphibian communities, driven by competition among saprophagous blowflies rather than host diversification *per se* (Arias-Robledo et al. 2019).

Our findings thus support multiple independent origins of parasitism in Luciliinae and the broader Calliphoridae, consistent with earlier studies (Stevens and Wall 1997; Stevens 2003; McDonagh and Stevens 2011). This pattern of rapid, repeated feeding-mode shifts in Luciliinae parallels the "episodic radiation" model proposed for Dipteran lineages (Wiegmann et al. 2011), in which short bursts of diversification (spanning ≤5 Ma) coincide with major environmental changes. We hypothesize that Miocene–Pliocene climatic and ecological shifts created transient ecological opportunities, driving multiple independent transitions in larval feeding habits over a relatively short evolutionary timescale.

Parasitic feeding habits in Luciliinae do not correspond strictly to geography but instead reflect phylogenetic history (Fig. 4), consistent with earlier findings (Williams et al. 2016). For instance, key myiasis-causing species (e.g., *L. cuprina*, *L.*

caesar, *L. illustris*, *L. sericata*) evolved independently in different clades, suggesting multiple origins of ectoparasitism during the late Miocene (Stevens and Wall 1996; Stevens and Wall 1997; Stevens 2003; Kapoor et al. 2025). These evolutionary events may be linked to ecological opportunity: for example, *L. sericata* is adapted to cooler temperate regions, while *L. cuprina* thrives in warmer climates, including subtropical zones (Stevens and Wall 1997). In *L. cuprina dorsalis*, myiasis is geographically restricted to areas such as Australia and South Africa, indicating that parasitism evolved in response to local host availability and environmental pressures (Stevens and Wall 1996; Stevens and Wall 1997; Stevens 2003; Kapoor et al. 2025). Conversely, most tropical lineages from Asia, Australasia, and the Afrotropics that diverged during the late Miocene remained saprophagous—likely due to the consistent availability of carrion resources in tropical habitats, reducing selective pressure for parasitic habits. Moreover, within the predominantly Nearctic clade that includes *L. albofusca*, *L. rica*, and *L. fayeae*, the most recent common ancestor is inferred to have been saprophagous. The evolution of parasitic traits appears to be highly labile in Luciliinae, with multiple independent transitions from saprophagy to facultative or obligate parasitism. Because of this flexibility, likelihood-based ancestral state reconstructions often identify saprophagy as the ancestral condition, especially when reversals or convergences are common (Zumpt 1965; Cardoso et al. 2025).

Finally, our analysis suggests that the evolution of parasitic feeding habits in blowflies is shaped more by ecological opportunity and evolutionary flexibility than by a single, progressive trajectory (e.g., from saprophagy to facultative to obligate parasitism). This dynamic pattern invites a reevaluation of simplistic models of parasitism evolution and highlights the need for broader taxonomic sampling beyond Luciliinae—especially using multiple molecular markers, including nuclear genes—to fully resolve ancestral states in ecologically versatile groups like Calliphoridae.

4. Conclusion

We present the most comprehensive mitogenomic and phylogenetic study of Luciliinae to date, resolving long-standing taxonomic uncertainties and reconstructing the evolutionary history of this medically, veterinarily and ecologically critical subfamily and lay a robust foundation for future morphological and genomic investigations. We confirm the paraphyly of *Lucilia*—with genera such as *Blepharicnema*, *Hemipyrellia*, and *Hypopygiopsis* nested within it—highlighting the urgent need for taxonomic

revision. This study is also the first to provide an estimated timeline of Luciliinae evolution and reconstruct the major historical events in biogeography and ecology across the subfamily, which have contributed to shape the extant diversity. Divergence dating reveals that Miocene geoclimatic shifts drove rapid diversification and repeated, independent origins of parasitic larval feeding within Luciliinae. Our findings challenge the traditional stepwise model of myiasis evolution (saprophagy → facultative parasitism → obligate parasitism), instead showing that facultative parasitism represents a flexible feeding habit from which lineages can shift toward or away from obligate host use depending on resource availability and host susceptibility.

Our results provide key insights into how changes in larval feeding habits are linked to species dispersal across regions and to the climatic shifts that occurred during the Miocene–Pliocene. Notably, transitions to obligate parasitism often coincided with major biogeographic events, as exemplified by the independent emergence of amphibian myiasis in the Nearctic and the Palearctic in *L. bufonivora* and *L. elongata*, ~4.2 Ma during the Pliocene, a period marked by intercontinental dispersal and significant climatic reorganization of amphibian habitats. Saprophagy remained dominant in Afrotropical lineages during early Miocene niche expansions into new decomposer guilds (e.g., carrion specialization in arid ecosystems). These spatiotemporal patterns underscore ecology as a driver of trophic evolution.

We identify tropical Africa as having been part of the early range of Luciliinae, consistent with the placement of *L. infernalis* as one of the two earliest splits (~13.1 Ma) within the lineage, although the precise geographic origin remains uncertain without formal biogeographic analysis. Our ancestral reconstructions favor saprophagy as the ancestral larval feeding habit of the lineage, with subsequent diversification into parasitic niches coinciding with Middle Miocene climatic changes, including grassland expansion and mammal host turnover.

Data Availability

The mitochondrial genome sequences generated in this study are available in NCBI, GenBank, and can be accessed with accession numbers provided in supplementary table S1, Supplementary Material online.

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Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT in order to improve readability. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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Figure Captions:

Fig. 1. Mitochondrial genome phylogeny of Luciliinae, based on 13 mitochondrial protein-coding genes (including all codon positions), using a maximum likelihood method in the IQ-TREE (ML123). The dataset includes mitogenomes of 50 species (79 taxa), including Ulurumyiidae as an outgroup. Nodal support is given as SH-alrt support/ IQ-TREE ultrafast bootstrap support. The voucher codes or Genbank accession numbers are included along with the species with more than one specimen, for clarity. Colors of the clades represent the corresponding calliphorid subfamilies as indicated in figure legends.

Fig. 2. Mitochondrial genome phylogeny of Luciliinae, based on 13 mitochondrial protein-coding genes (including all codon positions), using a Bayesian inference in ExaBayes (BI123). The dataset includes mitogenomes of 50 species (79 taxa), including Ulurumyiidae as an outgroup. Nodal support is given as ExaBayes posterior probabilities. The voucher codes or Genbank accession numbers are included along with the species with more than one specimen, for clarity. Colors of the clades represent the corresponding calliphorid subfamilies as indicated in figure legends.

Fig. 3. Divergence time estimation of Luciliinae and other calliphorids based on Bayesian uncorrelated relaxed clock model (two independent MCMCtree runs) with a Yule (pure birth) speciation model. Node values indicate mean estimated divergence times in million years (Ma) and blue bars represent 95% credibility intervals, numbers on scale represent million years ago. Calibration points for Oestroidea crown (O), Calliphoridae origin (C) and Calliphorinae + Luciliinae radiation (N) are shown in red

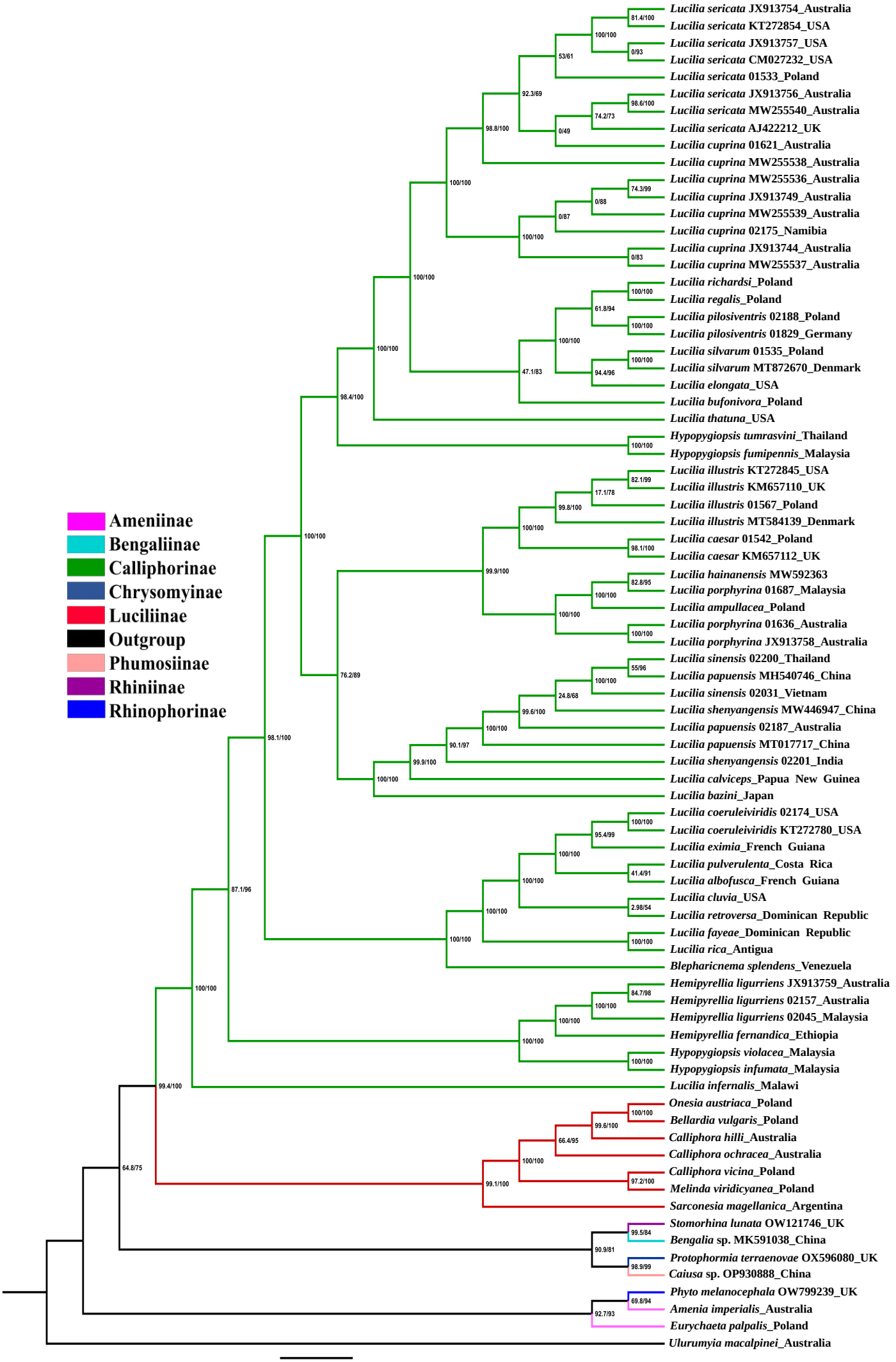
circles. Pliocene, Pleistocene, Holocene and Quaternary at geological time scale are shown as “Pli”, “Ple”, “H” and “Q.”, respectively.

Fig. 4. Ancestral state reconstruction of larval feeding habits in Calliphoridae, with an emphasis on Luciliinae using a maximum likelihood (ML) approach. Marginal reconstructions were performed with the fitMk function in phytools 2.0 (Revell, 2024) and ace in ape (Paradis, 2012) in R, based on an ultrametric time-calibrated phylogeny. Stochastic character mapping (SCM) was performed with “phytools::make.simmap” with 1000 MCMC simulations under a model-averaged framework. Circles at the tips indicate known feeding habits, specimens with six equally colored circles represent species with unknown larval feeding habit, modeled with equal prior probabilities across all six states. Pies at internal nodes represent the marginal probabilities of ancestral state.

Fig. S1. Geographic location of collection sites for specimens included in this study. Colored circles represent specimens from different calliphorid subfamilies and the outgroup (Ulurumyiidae) used in the phylogenetic analyses.

Fig. S2. Mitochondrial genome phylogeny of Luciliinae, based on 13 mitochondrial protein-coding genes (excluding the third codon), using a maximum likelihood method in the IQ-TREE (ML12). The dataset includes mitogenomes of 50 species (79 taxa), including Ulurumyiidae as an outgroup. Nodal support is given as SH-alrt support/ IQ-TREE ultrafast bootstrap support. The voucher codes or Genbank accession numbers are included along with the species with more than one specimen, for clarity.

Fig. S3. Mitochondrial genome phylogeny of Luciliinae, based on 13 mitochondrial protein-coding genes (excluding the third codon), using a Bayesian inference in ExaBayes (BI12). The dataset includes mitogenomes of 50 species (79 taxa), including Ulurumyiidae as an outgroup. Nodal support is given as ExaBayes posterior probabilities. The voucher codes or Genbank accession numbers are included along with the species with more than one specimen, for clarity.


Ameniinae
Bengaliinae
Calliphorinae
Chrysomyinae
Luciliinae
Outgroup
Phumosiinae
Rhiniinae
Rhinophorinae
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Lucilia sericata KT272854_USA
Lucilia sericata JX913757_USA
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Lucilia sericata 01533_Poland
Lucilia sericata JX913756_Australia
Lucilia sericata MW255540_Australia
Lucilia sericata AJ422212_UK
Lucilia cuprina 01621_Australia
Lucilia cuprina MW255538_Australia
Lucilia cuprina MW255536_Australia
Lucilia cuprina JX913749_Australia
Lucilia cuprina MW255539_Australia
Lucilia cuprina 02175_Namibia
Lucilia cuprina JX913744_Australia
Lucilia cuprina MW255537_Australia
Lucilia richardsi_Poland
Lucilia regalis_Poland
Lucilia pilosiventris 02188_Poland
Lucilia pilosiventris 01829_Germany
Lucilia silvarum 01535_Poland
Lucilia silvarum MT872670_Denmark
Lucilia elongata_USA
Lucilia bufonivora_Poland
Lucilia thatuna_USA
Hypopygiopsis tumrasvini_Thailand
Hypopygiopsis fumipennis_Malaysia
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Lucilia porphyrina 01687_Malaysia
Lucilia ampullacea_Poland
Lucilia porphyrina 01636_Australia
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Lucilia papuensis MT017717_China
Lucilia shenyangensis 02201_India
Lucilia calviceps_Papua New Guinea
Lucilia bazini_Japan
Lucilia coeruleiviridis 02174_USA
Lucilia coeruleiviridis KT272780_USA
Lucilia eximia_French Guiana
Lucilia pulverulenta_Costa Rica
Lucilia albofusca_French Guiana
Lucilia cluvia_USA
Lucilia retroversa_Dominican Republic
Lucilia fayeae_Dominican Republic
Lucilia rica_Antigua
Blepharicnema splendens_Venezuela
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Hypopygiopsis violacea_Malaysia
Hypopygiopsis infumata_Malaysia
Lucilia infernalis_Malawi
Onesia austriaca_Poland
Bellardia vulgaris_Poland
Calliphora hilli_Australia
Calliphora ochracea_Australia
Calliphora vicina_Poland
Melinda viridicyanea_Poland
Sarconesia magellanica_Argentina
Stomorhina lunata OW121746_UK
Bengalia sp. MK591038_China
Protophormia terraenovae OX596080_UK
Caiusa sp. OP930888_China
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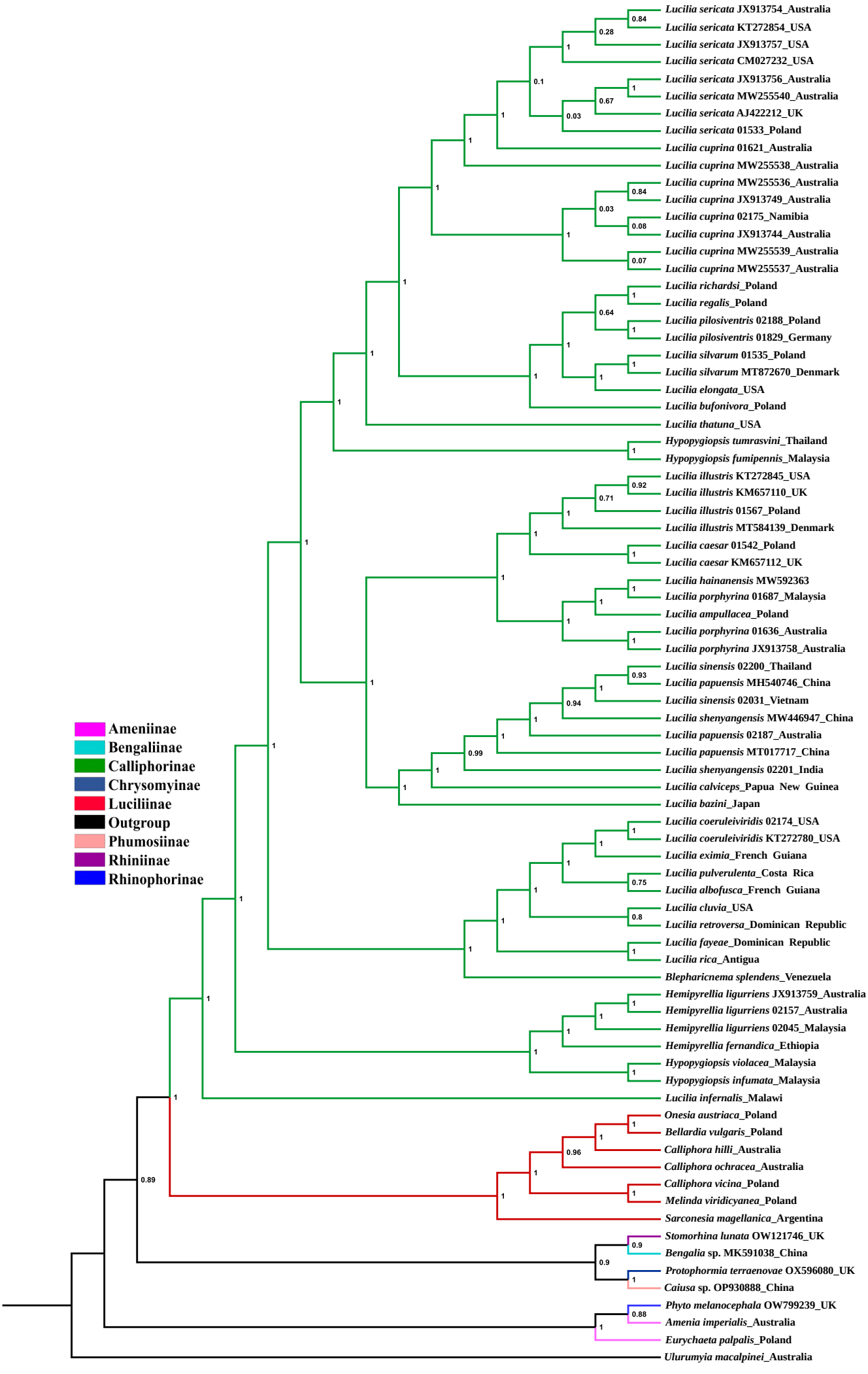
Fig. 1


Ameniinae
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Lucilia sericata JX913754_Australia
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Lucilia cuprina MW255538_Australia
Lucilia cuprina MW255536_Australia
Lucilia cuprina JX913749_Australia
Lucilia cuprina 02175_Namibia
Lucilia cuprina JX913744_Australia
Lucilia cuprina MW255539_Australia
Lucilia cuprina MW255537_Australia
Lucilia richardsi_Poland
Lucilia regalis_Poland
Lucilia pilosiventris 02188_Poland
Lucilia pilosiventris 01829_Germany
Lucilia silvarum 01535_Poland
Lucilia silvarum MT872670_Denmark
Lucilia elongata_USA
Lucilia bufonivora_Poland
Lucilia thatuna_USA
Hypopygiopsis tumrasvini_Thailand
Hypopygiopsis fumipennis_Malaysia
Lucilia illustris KT272845_USA
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Lucilia illustris MT584139_Denmark
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Lucilia ampullacea_Poland
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Lucilia papuensis MH540746_China
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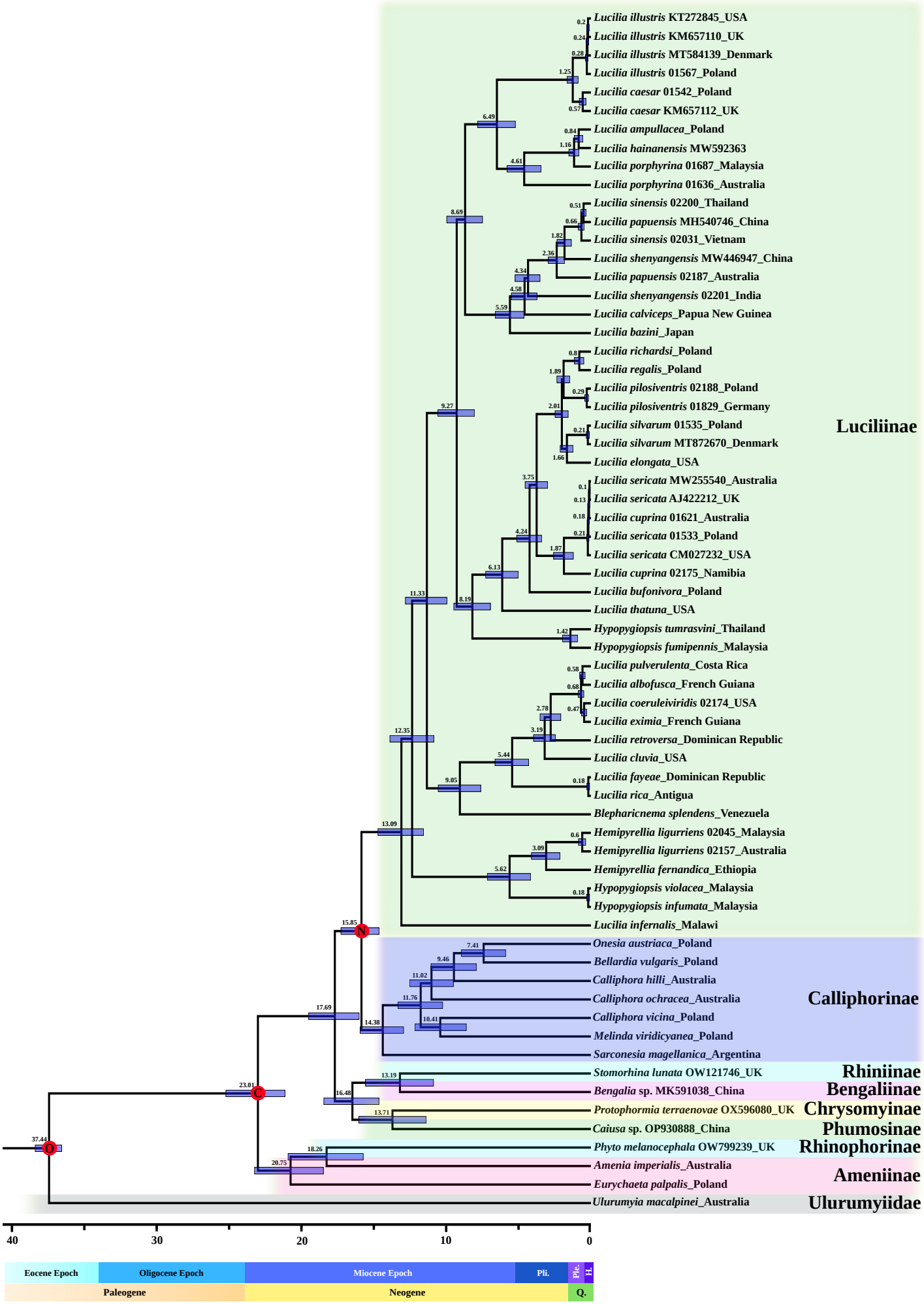
Lucilia papuensis 02187_Australia
Lucilia papuensis MT017717_China
Lucilia shenyangensis 02201_India
Lucilia calviceps_Papua New Guinea
Lucilia bazini_Japan
Lucilia coeruleiviridis 02174_USA
Lucilia coeruleiviridis KT272780_USA
Lucilia eximia_French Guiana
Lucilia pulverulenta_Costa Rica
Lucilia albofusca_French Guiana
Lucilia cluvia_USA
Lucilia retroversa_Dominican Republic
Lucilia fayeae_Dominican Republic
Lucilia rica_Antigua
Blepharicnema splendens_Venezuela
Hemipyrellia ligurriens JX913759_Australia
Hemipyrellia ligurriens 02157_Australia
Hemipyrellia ligurriens 02045_Malaysia
Hemipyrellia fernandica_Ethiopia
Hypopygiopsis violacea_Malaysia
Hypopygiopsis infumata_Malaysia
Lucilia infernalis_Malawi
Onesia austriaca_Poland
Bellardia vulgaris_Poland
Calliphora hilli_Australia
Calliphora ochracea_Australia
Calliphora vicina_Poland
Melinda viridicyanea_Poland
Sarconesia magellanica_Argentina
Stomorhina lunata OW121746_UK
Bengalia sp. MK591038_China
Protophormia terraenovae OX596080_UK
Caiusa sp. OP930888_China
Phyto melanocephala OW799239_UK
Amenia imperialis_Australia
Eurychaeta palpalis_Poland
Ulurumyia macalpinei_Australia
2.0

Fig. 2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Lucilia ampullacea_Poland
Lucilia hainanensis MW592363
Lucilia porphyrina 01687_Malaysia
Lucilia porphyrina 01636_Australia
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Lucilia papuensis 02187_Australia
Lucilia shenyangensis 02201_India
Lucilia calviceps_Papua New Guinea
Lucilia bazini_Japan
Lucilia richardsi_Poland
Lucilia regalis_Poland
Lucilia pilosiventris 02188_Poland
Lucilia pilosiventris 01829_Germany
Lucilia silvarum 01535_Poland
Lucilia silvarum MT872670_Denmark
Lucilia elongata_USA
Lucilia sericata MW255540_Australia
Lucilia sericata AJ422212_UK
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Lucilia sericata CM027232_USA
Lucilia cuprina 02175_Namibia
Lucilia bufonivora_Poland
Lucilia thatuna_USA
Hypopygiopsis tumrasvini_Thailand
Hypopygiopsis fumipennis_Malaysia
Lucilia pulverulenta_Costa Rica
Lucilia albofusca_French Guiana
Lucilia coeruleiviridis 02174_USA
Lucilia eximia_French Guiana
Lucilia retroversa_Dominican Republic
Lucilia cluvia_USA
Lucilia fayeae_Dominican Republic
Lucilia rica_Antigua
Blepharicnema splendens_Venezuela
Hemipyrellia ligurriens 02045_Malaysia
Hemipyrellia ligurriens 02157_Australia
Hemipyrellia fernandica_Ethiopia
Hypopygiopsis violacea_Malaysia
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Sarconesia magellanica_Argentina
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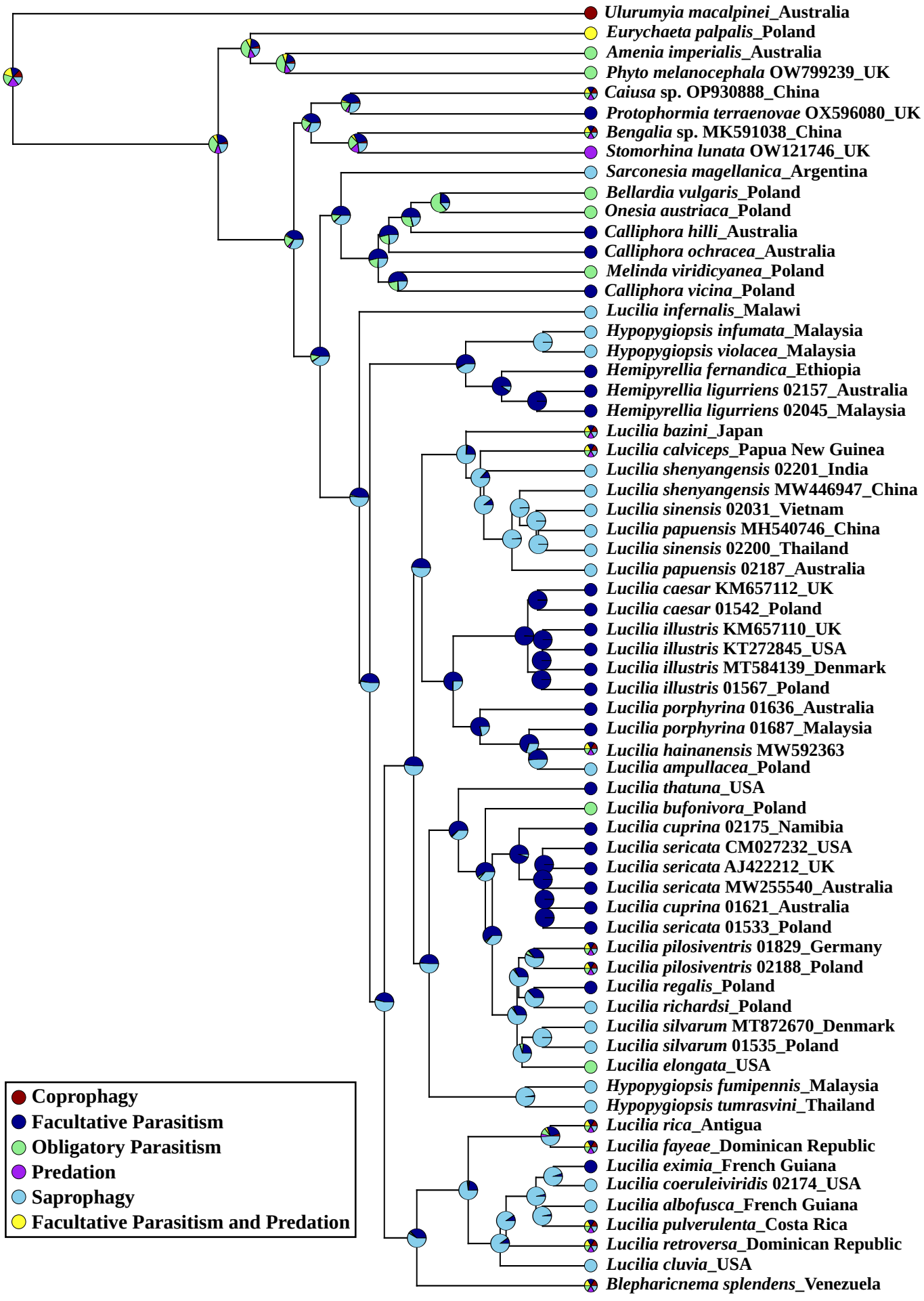
Protophormia terraenovae OX596080_UK
Caiusa sp. OP930888_China
Phyto melanocephala OW799239_UK
Amenia imperialis_Australia
Eurychaeta palpalis_Poland
Ulurumyia macalpinei_Australia
Luciliinae
Calliphorinae
Rhiniinae
Bengaliinae
Chrysomyinae
Phumosinae
Rhinophorinae
Ameniinae
Ulurumyiidae
O
C
N
40
30
20
10
0
Eocene Epoch
Oligocene Epoch
Miocene Epoch
Pli.
Ple.
H.
Paleogene
Neogene
Q.

Fig. 3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*Eurychaeta palpalis*_Poland
*Amenia imperialis*_Australia
Phyto melanocephala OW799239_UK
Caiusa sp. OP930888_China
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*Sarconesia magellanica*_Argentina
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*Lucilia bazini*_Japan
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Lucilia sericata 01533_Poland
Lucilia pilosiventris 01829_Germany
Lucilia pilosiventris 02188_Poland
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Lucilia silvarum MT872670_Denmark
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*Lucilia albofusca*_French Guiana
*Lucilia pulverulenta*_Costa Rica
*Lucilia retroversa*_Dominican Republic
*Lucilia cluvia*_USA
*Blepharicnema splendens*_Venezuela
Coprophagy
Facultative Parasitism
Obligatory Parasitism
Predation
Saprophagy
Facultative Parasitism and Predation

Fig. 4

Table S1. Summary of mitogenomic sequences used in this study, with Sequence ID, Geographic Origin, GenBank Accession Numbers, and Nucleotide Composition. A "•" and "*"represents the specimens sequenced in this study and species with partial mitochondrial genome, respectively.

											Base Composition					
ID	**Family**	**Subfamily**	**Genera**	**Species**	**Location**	**Latitude**	**Longitude**	**GenBank Accession**	**Source**	**Total length (bp)**	**T**	**C**	**A**	**G**	**AT-skew**	**GC-skew**
KEIB_DIP_02153	Calliphoridae	Luciliinae	*Blepharicnema*	*splendens*	Henri Pittier National Park,Venezuela	10.41	-67.61	PV604281	•	15020	37	14.5	38.6	9.9	0.0212	-0.1885
KEIB_DIP_02161	Calliphoridae	Luciliinae	*Hypopygiopsis*	*tumrasvini*	Chiang Mai Province, Doi Suthep NP, Thailand	18.81	98.89	PV604282	•	15334	38.1	13.4	39	9.5	0.0117	-0.1703
KEIB_DIP_02171	Calliphoridae	Luciliinae	*Lucilia*	*cluvia*	Flo, Okeechobee, USA	27.24	-80.82	PV604283	•	15095	37.6	14	38.8	9.7	0.0157	-0.1814
KEIB_DIP_02176	Calliphoridae	Luciliinae	*Lucilia*	*elongata*	OR, Klamath Falls, USA	42.22	-121.78	PV604284	•	15157	37.9	13.5	39	9.6	0.0143	-0.1688
KEIB_DIP_02178	Calliphoridae	Luciliinae	*Lucilia*	*fayeae*	Calibishe, Dominican Republic	15.58	-61.35	PV604285	•	14971	37	14.5	38.8	9.7	0.0237	-0.1983
KEIB_DIP_02180	Calliphoridae	Luciliinae	*Lucilia*	*infernalis*	Nichisi Forest, Malawi	-13.38	34.00	PV604286	•	14959	37.2	14.2	39	9.6	0.0236	-0.1933
KEIB_DIP_02187	Calliphoridae	Luciliinae	*Lucilia*	*papuensis*	QLD, Paluma Range NP, Australia	-19.09	146.27	PV604287	•	16004	37.6	13.4	39.7	9.3	0.0272	-0.1806
KEIB_DIP_02188	Calliphoridae	Luciliinae	*Lucilia*	*pilosiventris*	Skotniki Górne, Poland	50.42	20.64	PV604288	•	15428	37.6	13.9	38.9	9.7	0.017	-0.178
KEIB_DIP_02195	Calliphoridae	Luciliinae	*Lucilia*	*rica*	Antigua & Barbuda, Antigua	17.08	-61.79	PV604289	•	15835	37.4	14.1	39.2	9.3	0.0235	-0.2051
KEIB_DIP_02200	Calliphoridae	Luciliinae	*Lucilia*	*sinensis*	Ko Phi Phi, Thailand	7.74	98.78	PV604290	•	15360	37.4	13.7	39.6	9.3	0.0286	-0.1913
KEIB_DIP_02201	Calliphoridae	Luciliinae	*Lucilia*	*shenyangensis*	Utar Pradesh, Mussorie, India	30.46	78.06	PV604291	•	15364	37.4	13.6	39.6	9.4	0.0286	-0.1826
KEIB_DIP_02202	Calliphoridae	Luciliinae	*Lucilia*	*thatuna*	CA, Del Norte Co., Botanical Trial, USA	37.68	-106.35	PV604292	•	15026	37.7	13.8	38.9	9.7	0.0157	-0.1745
KEIB_DIP_02045	Calliphoridae	Luciliinae	*Hemipyrellia*	*ligurriens*	Pahang, Genting Sempah, Malaysia	3.39	101.78	PV604293	•	15913	37.9	13.2	39.6	9.3	0.0219	-0.1733

KEIB_DIP_01811	Calliphoridae	Luciliinae	*Hypopygiopsis*	*violacea*	Malaysia	2.02	102.98	PV604294	•	15384	37.3	13.9	39.2	9.5	0.0248	-0.188
KEIB_DIP_01685	Calliphoridae	Luciliinae	*Hypopygiopsis*	*fumipennis*	Pahang, Genting Sempah, Malaysia	3.39	101.78	PV604295	•	15939	38.4	12.9	39.3	9.4	0.0116	-0.157
KEIB_DIP_01676	Calliphoridae	Luciliinae	*Hypopygiopsis*	*infumata*	Pahang, Genting Sempah, Malaysia	3.39	101.78	PV604296	•	17089	37.2	14	39	9.8	0.0236	-0.1765
KEIB_DIP_01533	Calliphoridae	Luciliinae	*Lucilia*	*sericata*	Święta Katarzyna, Poland	50.90	20.88	PV604297	•	15430	38	13.3	39.2	9.4	0.0155	-0.1718
KEIB_DIP_01534	Calliphoridae	Luciliinae	*Lucilia*	*richardsi*	Święta Katarzyna, Poland	50.90	20.88	PV604298	•	15947	38.2	13.1	39.4	9.2	0.0155	-0.1749
KEIB_DIP_01535	Calliphoridae	Luciliinae	*Lucilia*	*silvarum*	Święta Katarzyna, Poland	50.90	20.88	PV604299	•	16138	38.3	13	39.4	9.3	0.0142	-0.1659
KEIB_DIP_01537	Calliphoridae	Luciliinae	*Lucilia*	*bufonivora*	Bartoszowiny, Poland	50.82	21.03	PV604300	•	15200	37.5	13.8	39.4	9.3	0.0247	-0.1948
KEIB_DIP_01538	Calliphoridae	Luciliinae	*Lucilia*	*ampullacea*	Jeleniów, Poland	50.83	21.1	PV604301	•	15970	37.5	13.9	39	9.6	0.0196	-0.183
KEIB_DIP_01567	Calliphoridae	Luciliinae	*Lucilia*	*illustris*	Złoty Potok, Poland	50.69	19.41	PV604302	•	15962	38	13.2	39.3	9.4	0.0168	-0.1681
KEIB_DIP_01621	Calliphoridae	Luciliinae	*Lucilia*	*cuprina*	Wollongong, UOW, Australia	-34.40	150.87	PV604303	•	16072	38.2	13.1	39.4	9.4	0.0155	-0.1644
KEIB_DIP_01687	Calliphoridae	Luciliinae	*Lucilia*	*porphyrina*	Pahang, Genting Sempah, Malaysia	3.39	101.78	PV604304	•	15931	37.6	13.8	39.2	9.4	0.0208	-0.1897
KEIB_DIP_01829	Calliphoridae	Luciliinae	*Lucilia*	*pilosiventris*	Germany	51.30	10.09	PV604305	•	15559	37.7	13.7	39	9.6	0.0169	-0.176
KEIB_DIP_01731	Calliphoridae	Luciliinae	*Lucilia*	*pulverulenta*	Puntarenas prov., Hacienda La Amistad, Costa Rica	8.93	-82.78	PV604306	•	16103	37.8	13.8	39.1	9.3	0.0169	-0.1948
KEIB_DIP_01828	Calliphoridae	Luciliinae	*Lucilia*	*regalis*	Nature Reserve "Zbocza Płutowskie", Poland	53.30	18.3733	PV604307	•	16889	38.2	13.1	38.9	9.8	0.0091	-0.1441
KEIB_DIP_02006	Calliphoridae	Luciliinae	*Lucilia*	*albofusca*	Amazone Nature Lodge, French Guina	4.558	-52.20	PV604308	•	16006	37.7	13.9	39.1	9.3	0.0182	-0.1983
KEIB_DIP_02031	Calliphoridae	Luciliinae	*Lucilia*	*sinensis*	Ninh Binh Province, Cuc Phuong NP, Vietnam	20.35	105.59	PV604309	•	15855	37.6	13.5	39.6	9.3	0.0259	-0.1842
KEIB_DIP_02165	Calliphoridae	Luciliinae	*Lucilia*	*bazini**	Okinawa, Nishihara, Japan	26.23	127.75	PV604334	•	13498	37	14.1	38.7	10.3	0.0225	-0.1557
KEIB_DIP_02170	Calliphoridae	Luciliinae	*Lucilia*	*calviceps**	New Ireland, Lemkamin, Papua New Guinea	-3.31	151.99	PV604335	•	13476	36.4	14.7	38.8	10.1	0.0319	-0.1855

KEIB_DIP_02175	Calliphoridae	Luciliinae	*Lucilia*	*cuprina**	Homeb, Namib-Naukluft NP, Namibia	-23.54	15.29	PV604336	•	13594	37.6	13.6	38.7	10	0.0144	-0.1525
KEIB_DIP_01542	Calliphoridae	Luciliinae	*Lucilia*	*caesar*	Jeleniów, Poland	50.83	21.10	PV604337	•	15950	38	13.2	39.3	9.4	0.0168	-0.1681
KEIB_DIP_02174	Calliphoridae	Luciliinae	*Lucilia*	*coeruleiviridis**	Missouri, Putnam, Martinstown, USA	40.41	-92.77	PV604341	•	14915	37.3	14.3	38.6	9.7	0.0171	-0.1917
KEIB_DIP_02157	Calliphoridae	Luciliinae	*Hemipyrellia*	*ligurriens**	Holmes Jungle Nat.Park, Australia	-12.39	130.93	Appendix 1	•	NA						
KEIB_DIP_02194	Calliphoridae	Luciliinae	*Lucilia*	*retroversa**	Hato Mayor, Dominican Republic	18.80	-69.31	Appendix 1	•	NA						
PQ593989	Calliphoridae	Calliphorinae	*Calliphora*	*vicina*	Święta Katarzyna, Poland	50.90	20.88	PQ593989	GenBank	16386	38.1	13	39.5	9.3	0.018	-0.1659
PQ593996	Calliphoridae	Calliphorinae	*Calliphora*	*ochracea*	Seven Mile Beach, Australia	-34.79	150.77	PQ593996	GenBank	16554	38.2	12.8	39.8	9.1	0.0205	-0.1689
PQ594000	Calliphoridae	Calliphorinae	*Calliphora*	*hilli*	Wollongong, Mt. Keira, Australia	-34.40	150.85	PQ594000	GenBank	16414	39.6	9.4	37.6	13.4	-0.0259	0.1754
PQ635409	Calliphoridae	Calliphorinae	*Onesia*	*austriaca*	"Góry Pieprzowe" Nature Reserve, Poland	50.68	21.79	PQ635409	GenBank	14904	39.8	9.4	38.2	12.7	-0.0205	0.1493
PQ664927	Calliphoridae	Calliphorinae	*Bellardia*	*vulgaris*	Święta Katarzyna, Poland	50.90	20.88	PQ664927	GenBank	16216	40	9.2	37.8	13	-0.0283	0.1712
PQ664953	Calliphoridae	Calliphorinae	*Melinda*	*viridicyanea*	Kamieniolom Kantyna, Poland	50.89	16.69	PQ664953	GenBank	15052	38.6	12.3	40.2	9	0.0203	-0.1549
PQ649436	Calliphoridae	Calliphorinae	*Sarconesia*	*magellanica*	Salta Prov., 2 km W Cafayate, Argentina	-26.07	-65.89	PQ649436	GenBank	15079	37.7	13.2	39.3	9.7	0.0208	-0.1528
PQ649437	Calliphoridae	Luciliinae	*Hemipyrellia*	*fernandica*	Arba Minch, Nechisar NP, Ethiopia	6.01	37.55	PQ649437	GenBank	16127	38	13.2	39.6	9.1	0.0206	-0.1839
PQ649438	Calliphoridae	Luciliinae	*Lucilia*	*porphyrina*	Macquire Pass, Australia	-34.56	150.67	PQ649438	GenBank	15874	37.2	14.2	39	9.6	0.0236	-0.1933
PQ649439	Calliphoridae	Luciliinae	*Lucilia*	*eximia*	Chutes Fourgassier, French Guiana	4.62	-52.30	PQ649439	GenBank	15990	37.8	13.8	39.1	9.3	0.0169	-0.1948
PQ665281	Calliphoridae	Ameniinae	*Amenia*	*imperialis*	Berrara Creek, Australia	-35.20	150.53	PQ665281	GenBank	16198	37.8	13.5	39.2	9.6	0.0182	-0.1688
PQ665283	Calliphoridae	Ameniinae	*Eurychaeta*	*palpalis*	Zatwarnica, Poland	49.22	22.55	PQ665283	GenBank	16593	38.6	12.6	39.9	8.8	0.0166	-0.1776
PQ654165	Ulurumyiidae		*Ulurumyia*	*macalpinei*	NSW, Blue Mts, Megalong Valley, Australia	-33.65	150.27	PQ654165	GenBank	15140	39.1	9.6	37.2	14.2	-0.0249	0.1933

MK591038	Calliphoridae	Bengaliinae	*Bengalia*	sp.	Jianfengling National Forest Park, Hainan province, China	18.70	108.92	MK591038	GenBank	15784	37.7	13.7	39.3	9.3	0.0208	-0.1913
OW121746	Calliphoridae	Rhiniinae	*Stomorhina*	*lunata*	Oxfordshire, Hartslock Reserve, UK	51.51	-1.11	OW121746	GenBank	16491	40.4	8.9	38.4	12.3	-0.0254	0.1604
OW799239	Calliphoridae	Rhinophorinae	*Phyto*	*melanocephala*	Hartslock, England, UK	51.51	-1.11	OW799239	GenBank	16280	41.5	8.1	38.8	11.6	-0.0336	0.1777
OP930888	Calliphoridae	Phumosinae	*Caiusa*	sp.	China	NA	NA	OP930888	GenBank	14911	37.6	13.8	39	9.6	0.0183	-0.1795
OX596080	Calliphoridae	Chrysomyinae	*Protophormia*	*terraenovae*	Drumjohn Power Station, Dumfried and Galloway, UK	55.25	-4.32	OX596080	GenBank	15752	39.1	9.5	37.4	14	-0.0222	0.1915
CM027232	Calliphoridae	Luciliinae	*Lucilia*	*sericata*	Raleigh, North Carolina, USA	NA	NA	CM027232	GenBank	15961	39.3	9.5	38.2	13.1	-0.0142	0.1593
MW255540	Calliphoridae	Luciliinae	*Lucilia*	*sericata*	Campbell Town, Tasmania, Australia	-41.92	147.48	MW255540	GenBank	15946	38.2	13	39.4	9.3	0.0155	-0.1659
AJ422212	Calliphoridae	Luciliinae	*Lucilia*	*sericata*	Bristol, UK	NA	NA	AJ422212	GenBank	15945	38.2	13	39.4	9.3	0.0155	-0.1659
JX913757	Calliphoridae	Luciliinae	*Lucilia*	*sericata*	Brigham Young University campus, Provo, UT, USA	NA	NA	JX913757	GenBank	15380	38.1	13.3	39.1	9.5	0.013	-0.1667
JX913754	Calliphoridae	Luciliinae	*Lucilia*	*sericata*	Canberra, ACT, Australia	NA	NA	JX913754	GenBank	15243	38	13.4	39.1	9.5	0.0143	-0.1703
JX913756	Calliphoridae	Luciliinae	*Lucilia*	*sericata*	Perth, WA, Australia	NA	NA	JX913756	GenBank	15214	38	13.4	39	9.5	0.013	-0.1703
KT272854	Calliphoridae	Luciliinae	*Lucilia*	*sericata*	University Park, PA, USA	NA	NA	KT272854	GenBank	15092	37.9	13.4	39	9.7	0.0143	-0.1602
JX913744	Calliphoridae	Luciliinae	*Lucilia*	*cuprina*	University of Melbourne colony, Australia	NA	NA	JX913744	GenBank	15952	38.1	13	39.5	9.3	0.018	-0.1659
MW255538	Calliphoridae	Luciliinae	*Lucilia*	*cuprina*	Brisbane, Queensland, Australia	-27.46	153.02	MW255538	GenBank	15952	38.2	13.1	39.4	9.3	0.0155	-0.1696
MW255539	Calliphoridae	Luciliinae	*Lucilia*	*cuprina*	Mount Romance, Western Australia, Australia	-34.76	117.13	MW255539	GenBank	15944	38.2	13	39.5	9.3	0.0167	-0.1659
MW255536	Calliphoridae	Luciliinae	*Lucilia*	*cuprina*	Carwarp, Victoria, Australia	-36.71	142.19	MW255536	GenBank	15941	38.2	13	39.5	9.3	0.0167	-0.1659
MW255537	Calliphoridae	Luciliinae	*Lucilia*	*cuprina*	McMahons Reef, New South Wales, Australia	-34.65	148.45	MW255537	GenBank	15941	38.2	13	39.5	9.3	0.0167	-0.1659
JX913749	Calliphoridae	Luciliinae	*Lucilia*	*cuprina*	Petrie Terrace, Brisbane, Qld, Australia	NA	NA	JX913749	GenBank	15348	38	13.3	39.2	9.5	0.0155	-0.1667

KM657112	Calliphoridae	Luciliinae	*Lucilia*	*caesar*	Burnley, UK	53.76	−2.23	KM657112	GenBank	15957	38	13.3	39.3	9.4	0.0168	-0.1718
KM657110	Calliphoridae	Luciliinae	*Lucilia*	*illustris*	Burnley, UK	53.76	−2.23	KM657110	GenBank	15956	38	13.2	39.4	9.4	0.0181	-0.1681
KT272845	Calliphoridae	Luciliinae	*Lucilia*	*illustris*	University Park, PA, USA	NA	NA	KT272845	GenBank	14875	37.8	13.7	38.7	9.8	0.0118	-0.166
MT584139	Calliphoridae	Luciliinae	*Lucilia*	*illustris*	Denmark	NA	NA	MT584139	GenBank	14527	37.7	13.8	38.5	9.9	0.0105	-0.1646
MH540746	Calliphoridae	Luciliinae	*Lucilia*	*papuensis*	China	22.65	99.6	MH540746	GenBank	15884	37.4	13.6	39.6	9.3	0.0286	-0.1878
MT017717	Calliphoridae	Luciliinae	*Lucilia*	*papuensis*	Beijing, China	35.4	116.57	MT017717	GenBank	15323	37.4	13.7	39.5	9.4	0.0273	-0.1861
JX913758	Calliphoridae	Luciliinae	*Lucilia*	*porphyrina*	University of Queensland campus, St Lucia, Brisbane, Qld, Australia	NA	NA	JX913758	GenBank	15877	37.2	14.2	39.1	9.5	0.0249	-0.1983
MT872670	Calliphoridae	Luciliinae	*Lucilia*	*silvarum*	Denmark	NA	NA	MT872670	GenBank	15517	38	13.2	39.2	9.5	0.0155	-0.163
MW592363	Calliphoridae	Luciliinae	*Lucilia*	*hainanensis*	NA	NA	NA	MW592363	GenBank	15319	37.3	14.2	38.8	9.7	0.0197	-0.1883
KT272780	Calliphoridae	Luciliinae	*Lucilia*	*coeruleiviridis*	University Park, PA, USA	NA	NA	KT272780	GenBank	14989	37.4	14.2	38.7	9.7	0.0171	-0.1883
MW446947	Calliphoridae	Luciliinae	*Lucilia*	*shenyangensis*	Beijing, China	39.8	116.46	MW446947	GenBank	14989	37.3	14	39.2	9.6	0.0248	-0.1864
JX913759	Calliphoridae	Luciliinae	*Hemipyrellia*	*ligurriens*	University of Queensland campus, St Lucia, Brisbane, Qld, Australia	NA	NA	JX913759	GenBank	15938	37.8	13.3	39.5	9.3	0.022	-0.177

Appendix 1

			GenBank Accession												
			ATP6	**ATP8**	**COI**	**COII**	**COIII**	**CYTB**	**ND1**	**ND2**	**ND3**	**ND4**	**ND4L**	**ND5**	**ND6**
KEIB_DIP_02157	*Hemipyrellia*	*ligurriens**	PV593855	PV593860	PV591018	PV593865	PV593870	PV593875	PV593880	PV593885	PV593890	PV593895	PV593900	PV593905	PV593910
KEIB_DIP_02194	*Lucilia*	*retroversa**	PV593856	PV593861	PV591019	PV593866	PV593871	PV593876	PV593881	PV593886	PV593891	PV593896	PV593901	PV593906	PV593911

Table S2. The optimal evolutionary models for all codon positions of PCGs (PCG123), calculated by IQ-TREE according to the BIC for ML analysis.

#nexus
begin sets;
charset atp6_pos1_cox3_pos1_cox2_pos1_cox1_pos1_cytb_pos1_nd6_pos2 = 1-678\3 844-1632\3 1633-2320\3 2321-3859\3 3860-4996\3 10682-11205\3;
charset atp6_pos2_cox3_pos2_cox2_pos2_cox1_pos2_cytb_pos2_nd3_pos2 = 2-678\3 845-1632\3 1634-2320\3 2322-3859\3 3861-4996\3 6963-7318\3;
charset
atp6_pos3_atp8_pos3_cox3_pos3_cox2_pos3_cox1_pos3_cytb_pos3_nd2_pos3_nd3_pos3_nd6_pos3 = 3-678\3 681-843\3 846-1632\3 1635-2320\3 2323-3859\3 3862-4996\3 5947-6961\3 6964-7318\3 10683-11205\3;
charset atp8_pos1_nd2_pos1_nd3_pos1_nd6_pos1 = 679-843\3 5945-6961\3 6962-7318\3 10681-11205\3;
charset atp8_pos2_nd1_pos2_nd2_pos2_nd4l_pos2_nd4_pos2_nd5_pos2 = 680-843\3 4998-5944\3 5946-6961\3 7320-7615\3 7617-8954\3 8956-10680\3;
charset nd1_pos1_nd4l_pos1_nd4_pos1_nd5_pos1 = 4997-5944\3 7319-7615\3 7616-8954\3 8955-10680\3;
charset nd1_pos3_nd4l_pos3_nd4_pos3_nd5_pos3 = 4999-5944\3 7321-7615\3 7618-8954\3 8957-10680\3;
charpartition mymodels =
TIM2+F+I+I+R3:
atp6_pos1_cox3_pos1_cox2_pos1_cox1_pos1_cytb_pos1_nd6_pos2,
TPM3u+F+I+I+R2:
atp6_pos2_cox3_pos2_cox2_pos2_cox1_pos2_cytb_pos2_nd3_pos2,
TIM2+F+I+I+R6:
atp6_pos3_atp8_pos3_cox3_pos3_cox2_pos3_cox1_pos3_cytb_pos3_nd2_pos3_nd3_pos3_nd6_pos3,
TIM2+F+I+G4: atp8_pos1_nd2_pos1_nd3_pos1_nd6_pos1,
TVM+F+I+I+R3: atp8_pos2_nd1_pos2_nd2_pos2_nd4l_pos2_nd4_pos2_nd5_pos2,
TIM2+F+I+I+R3: nd1_pos1_nd4l_pos1_nd4_pos1_nd5_pos1,
TIM+F+I+I+R5: nd1_pos3_nd4l_pos3_nd4_pos3_nd5_pos3;
end;

Table S3. Calibration points used for divergence time estimation in BEAST analysis, based on the previous estimation in Oestroidea (Junqueira et al., 2016).

1. The Oestroidea crown

The earliest known Oestridae fossils are from the Eocene shale deposits of the Green River Formation in North America. Biogeographic evidence supports the origin of Oestroidea during the same period[1].

Previous studies have dated the diversification of Oestroidea to the late Eocene, approximately 37.65 Ma (95% CI: 35–40 Ma)[2], which aligns with estimates by Wiegmann et al.[3]. Based on these findings, we applied a minimum age constraint of 35 million years (Ma) and a maximum of 60 Ma for the Oestroidea clade.

2. The Calliphoridae

Mitogenomic data indicate that the most recent common ancestor of the core Calliphoridae lineage emerged near the Oligocene–Miocene boundary, around 22.4 Ma, as reported by Junqueira et al. (2016). Wiegmann et al.[3] provided a comparable estimate of 21.7 Ma. Since our dataset closely matches that of Junqueira et al.[2], we used their estimate as the basis for calibration. We applied a minimum age of 20 Ma and a maximum of 25 Ma for the Calliphoridae clade.

3. The Calliphorinae + Luciliinae clade

Following the divergence within Calliphoridae, a rapid diversification event gave rise to the sister subfamilies Calliphorinae and Luciliinae, estimated to have occurred around 16.32 Ma, according to Junqueira et al.[2]. This estimate is consistent with that of Wiegmann et al.[3]. In our study, we constrained the node representing this split with a minimum age of 14 Ma and a maximum of 20 Ma.

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Table S4. Larval habits of species based on earlier studies, literature and expert knowledge, coded as discrete characters in Ancestral state reconstruction. Larval biology for many species of Luciliinae is still unclear or unknown and are labelled as NA. Cited literatures are listed as Appendix 1.

Species	Trait	Reference
Amenia imperialis	Obligatory parasitism	Clark 2009[1]
Bellardia vulgaris	Obligatory parasitism	Prado e Castro et al. 2016[2]
Bengalia sp.	NA	
Blepharicnema splendens	Sparophagous?? (Coded as NA)	Amat and Wolff 2007[3]
Caiusa sp.	NA	
Calliphora hilli	Facultative parasitism	Farrell et al. 2015[4], Fuller 1931[5]
Calliphora ochracea	Facultative parasitism	Farrell et al. 2015[4], Fuller 1931[5]
Calliphora vicina	Facultative parasitism	McDonagh and Stevens 2011[6], Limsopatham et al. 2018[7]
Eurychaeta palpalis	Facultative parasitism and Predation	Falk 2016[8]
Hemipyrellia fernandica	Facultative parasitism	De Jong and Krell 2011[9]
Hemipyrellia ligurriens	Facultative parasitism	Sukontason et al. 2008[10], Sinha 2012[11]
Hypopygiopsis fumipennis	Saprophagy	Nordin et al. 2020[12]
Hypopygiopsis infumata	Saprophagy	Sanit et al. 2018[13]
Hypopygiopsis tumrasvini	Saprophagy	Sanit et al. 2012[14]
Hypopygiopsis violacea	Saprophagy	Chen et al. 2011[15], Ahmad Firdaus et al. 2010[16]
Lucilia coeruleiviridis	Saprophagy	Weidner et al. 2014[17]
Lucilia albofusca	Saprophagy	Whitworth 2014[18]
Lucilia ampullacea	Saprophagy	Vanin et al. 2008[19]
Lucilia bazini	NA	
Lucilia bufonivora	Obligatory parasitism	Stevens and Wall 1996[20], Arias-Robledo et al. 2019[21]
Lucilia caesar	Facultative parasitism	Stevens and Wall 1996[20]
Lucilia calviceps	NA	
Lucilia cluvia	Saprophagy	Hall 1948[22], Zumpt[23]
Lucilia cuprina	Facultative parasitism	Stevens & Wall[20], Anderson et al.[24]
Lucilia elongata	Obligatory parasitism	Williams et al.[25]
Lucilia eximia	Facultative parasitism	Sanford et al.[26]
Lucilia fayeae	Sparophagous?? (Coded as NA)	Whitworth 2014[18]
Lucilia hainanensis	NA	

Lucilia illustris	Facultative parasitism	Zumpt[23], Wang et al.[27]
Lucilia infernalis	Saprophagy	Hall[22], Zumpt 1965[23]
Lucilia papuensis	Saprophagy	Hall[22], Zumpt 1965[23]
Lucilia pilosiventris	NA	
Lucilia porphyrina	Facultative parasitism	Zumpt 1965[23]
Lucilia pulverulenta	NA	
Lucilia regalis	Facultative parasitism	Arias-Robledo et al. 2019[21]
Lucilia retroversa	NA	
Lucilia rica	NA	
Lucilia richardsi	Saprophagy	Stevens and Wall 1997[28]
Lucilia sericata	Facultative parasitism	Stevens and Wall 1996[20]
Lucilia shenyangensis	Saprophagy	Xue and Zhao 1996[29]
Lucilia silvarum	Saprophagy	Arias-Robledo et al. 2019[21]
Lucilia sinensis	Saprophagy	Sanit et al. 2017[30]
Lucilia thatuna	Facultative parasitism	Tantawi and Whitworth 2014[31]
Melinda viridicyanea	Obligatory parasitism	Prado e Castro et al. 2016[2]
Onesia austriaca	Obligatory parasitism	Prado e Castro et al. 2016[2], McDonagh and Stevens 2011[6]
Phyto melanocephala	Obligatory parasitism	Cerretti comm. pers.
Protophormia terraenovae	Facultative parasitism	Warren and Anderson 2013[32]
Sarconesia magellanica	Saprophagy	Segura et al. 2009[33]
Stomorhina lunata	Predation	Thomas-Cabianca et al. 2023[34]
Ulurumyia macalpinei	Coprophagy	Michelsen and Pape 2017[35], Cerretti comm. pers

Appendix 1

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Table S5. Nucleotide composition of Luciliinae mitogenomes.

Gene	Feature	*Blepharicnema*	*Hemipyrellia*	*Hypopygiopsis*	*Lucilia*	Luciliinae
Entire mitochondrial genome	A+T %	75.6	77.466	76.875	76.89	76.89
	G+C %	24.4	22.466	23.1	23.1	23.09
	AT-skew	0.021	0.021	0.018	0.018	0.018
	GC-skew	-0.19	-0.18	-0.17	-0.17	-0.171
Protein-coding genes	A+T %	74.44	75.65	75.58	75.47	75.475
	G+C %	25.56	24.35	24.42	24.53	24.525
	AT-skew	-0.16	-0.16	-0.153	-0.157	-0.157
	GC-skew	0.014	0.027	0.021	0.022	0.022

Table S6. Approximately unbiased (AU) topology tests for 10,000 replicates under the model estimated by IQ-TREE

Tree	**logL**	**bp-RELL**	**deltaL**	**p-KH**	**p-SH**	**p-AU**
1 (Unconstrained - Polyphyletic *Lucilia*)	-100246.515	0.506 +	0	1 +	1 +	1 +
2 (Constrained - Monophyletic *Lucilia*)	-100246.601	0.494 +	0.086341	0 -	0 -	4.55e-11 -

deltaL: logL difference from the maximal logl in the set

bp-RELL: bootstrap proportion using RELL method (Kishino et al. 1990).

p-KH: p-value of one sided Kishino-Hasegawa test (1989).

p-SH: p-value of Shimodaira-Hasegawa test (2000).

p-AU: p-value of approximately unbiased (AU) test (Shimodaira, 2002).

Plus (+) sign denote the 95% confidence sets.

Minus (-) sign denote significant exclusion.

All tests performed 10000 resampling using the RELL method.

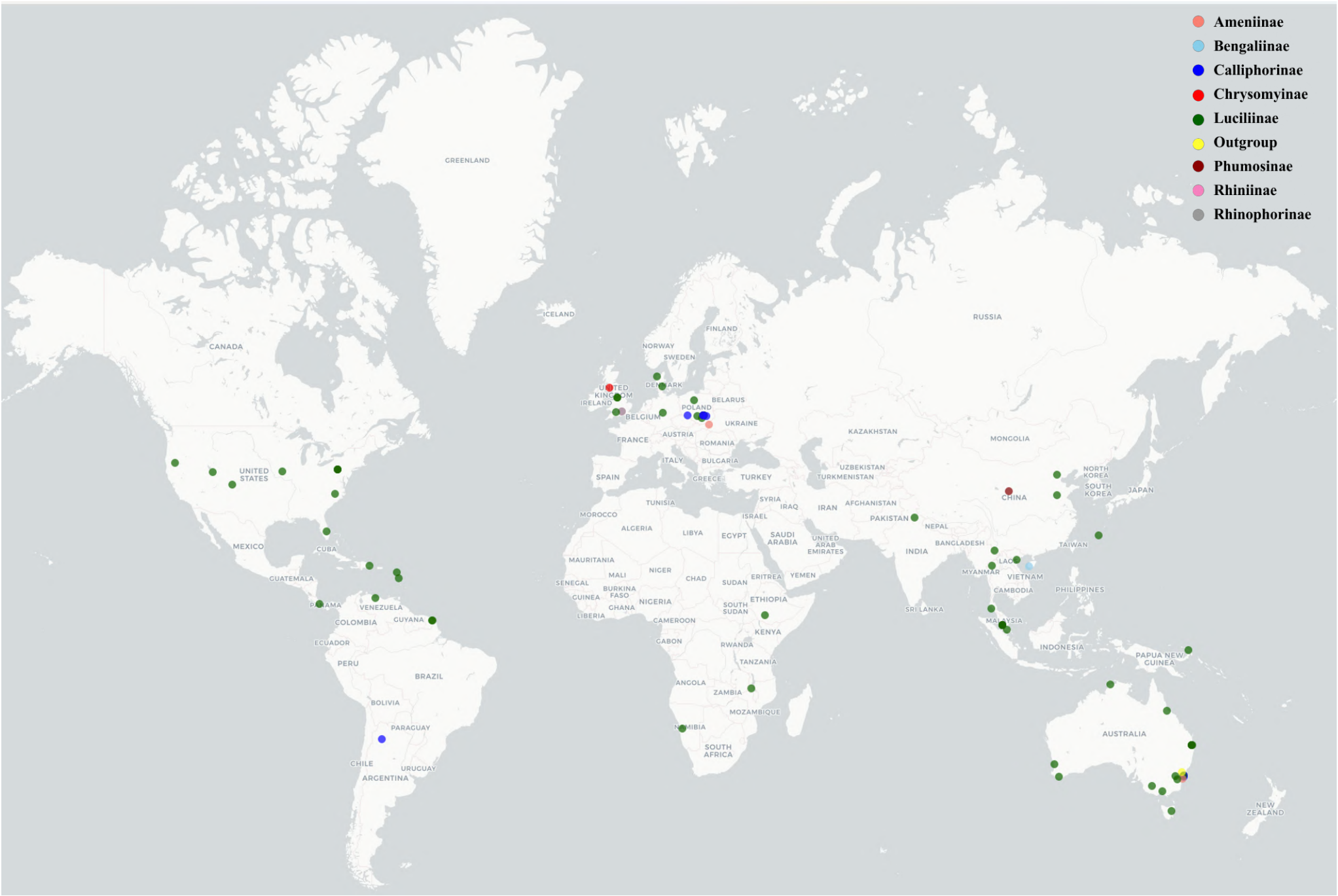
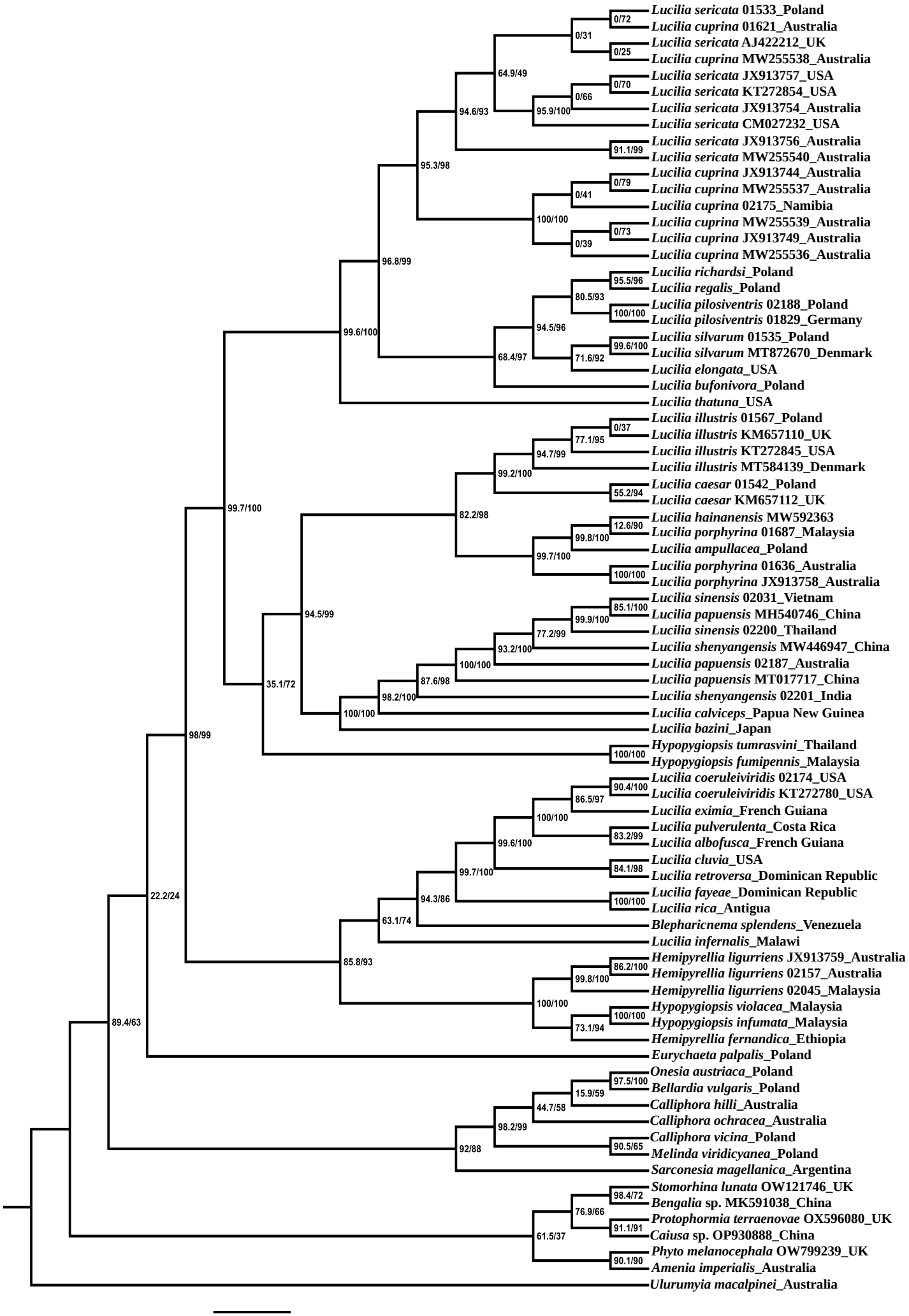

Ameniinae
Bengaliinae
Calliphorinae
Chrysomyinae
Luciliinae
Outgroup
Phumosinae
Rhiniinae
Rhinophorinae
GREENLAND
ICELAND
CANADA
UNITED STATES
MEXICO
CUBA
GUATEMALA
VENEZUELA
COLOMBIA
GUYANA
ECUADOR
PERU
BRAZIL
BOLIVIA
PARAGUAY
CHILE
URUGUAY
ARGENTINA
FINLAND
NORWAY
SWEDEN
DENMARK
BELARUS
POLAND
BELGIUM
UKRAINE
AUSTRIA
FRANCE
ROMANIA
ITALY
BULGARIA
SPAIN
GREECE
TURKEY
TUNISIA
SYRIA
IRAQ
IRAN
MOROCCO
ALGERIA
LIBYA
EGYPT
ISRAEL
SAUDI ARABIA
UNITED ARAB EMIRATES
MAURITANIA
MALI
NIGER
CHAD
SUDAN
ERITREA
YEMEN
SENEGAL
BURKINA FASO
GUINEA
NIGERIA
GHANA
LIBERIA
CAMEROON
SOUTH SUDAN
ETHIOPIA
KENYA
GABON
RWANDA
TANZANIA
ANGOLA
ZAMBIA
MOZAMBIQUE
NAMIBIA
SOUTH AFRICA
RUSSIA
KAZAKHSTAN
MONGOLIA
UZBEKISTAN
TURKMENISTAN
AFGHANISTAN
PAKISTAN
NEPAL
INDIA
BANGLADESH
SRI LANKA
CHINA
NORTH KOREA
SOUTH KOREA
JAPAN
TAIWAN
LAOS
MYANMAR
VIETNAM
CAMBODIA
PHILIPPINES
MALAYSIA
INDONESIA
PAPUA NEW GUINEA
AUSTRALIA
NEW ZEALAND

Fig. S1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Lucilia sericata CM027232_USA
Lucilia sericata JX913756_Australia
Lucilia sericata MW255540_Australia
Lucilia cuprina JX913744_Australia
Lucilia cuprina MW255537_Australia
Lucilia cuprina 02175_Namibia
Lucilia cuprina MW255539_Australia
Lucilia cuprina JX913749_Australia
Lucilia cuprina MW255536_Australia
Lucilia richardsi_Poland
Lucilia regalis_Poland
Lucilia pilosiventris 02188_Poland
Lucilia pilosiventris 01829_Germany
Lucilia silvarum 01535_Poland
Lucilia silvarum MT872670_Denmark
Lucilia elongata_USA
Lucilia bufonivora_Poland
Lucilia thatuna_USA
Lucilia illustris 01567_Poland
Lucilia illustris KM657110_UK
Lucilia illustris KT272845_USA
Lucilia illustris MT584139_Denmark
Lucilia caesar 01542_Poland
Lucilia caesar KM657112_UK
Lucilia hainanensis MW592363
Lucilia porphyrina 01687_Malaysia
Lucilia ampullacea_Poland
Lucilia porphyrina 01636_Australia
Lucilia porphyrina JX913758_Australia
Lucilia sinensis 02031_Vietnam
Lucilia papuensis MH540746_China
Lucilia sinensis 02200_Thailand
Lucilia shenyangensis MW446947_China
Lucilia papuensis 02187_Australia
Lucilia papuensis MT017717_China
Lucilia shenyangensis 02201_India
Lucilia calviceps_Papua New Guinea
Lucilia bazini_Japan
Hypopygiopsis tumrasvini_Thailand
Hypopygiopsis fumipennis_Malaysia
Lucilia coeruleiviridis 02174_USA
Lucilia coeruleiviridis KT272780_USA
Lucilia eximia_French Guiana
Lucilia pulverulenta_Costa Rica
Lucilia albofusca_French Guiana
Lucilia cluvia_USA
Lucilia retroversa_Dominican Republic
Lucilia fayeae_Dominican Republic
Lucilia rica_Antigua
Blepharicnema splendens_Venezuela
Lucilia infernalis_Malawi
Hemipyrellia ligurriens JX913759_Australia
Hemipyrellia ligurriens 02157_Australia
Hemipyrellia ligurriens 02045_Malaysia
Hypopygiopsis violacea_Malaysia
Hypopygiopsis infumata_Malaysia
Hemipyrellia fernandica_Ethiopia
Eurychaeta palpalis_Poland
Onesia austriaca_Poland
Bellardia vulgaris_Poland
Calliphora hilli_Australia
Calliphora ochracea_Australia
Calliphora vicina_Poland
Melinda viridicyanea_Poland
Sarconesia magellanica_Argentina
Stomorhina lunata OW121746_UK
Bengalia sp. MK591038_China
Protophormia terraenovae OX596080_UK
Caiusa sp. OP930888_China
Phyto melanocephala OW799239_UK
Amenia imperialis_Australia
Ulurumyia macalpinei_Australia
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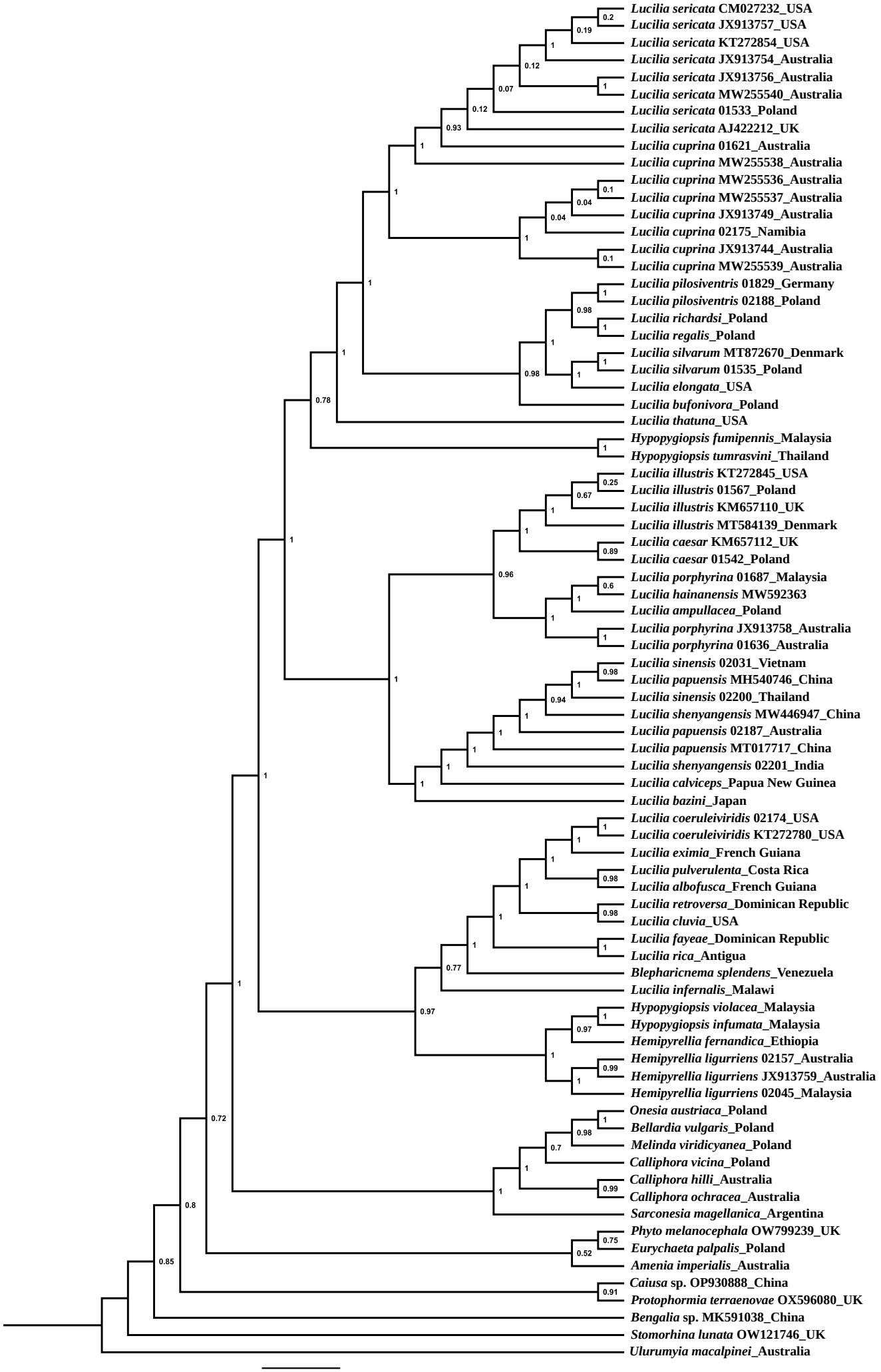
Fig. S2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Bengalia sp. MK591038_China
Stomorhina lunata OW121746_UK
*Ulurumyia macalpinei*_Australia
3.0

Fig. S3

Though written far from home, this work is deeply connected to the roots, values, and inspiration I carry from my homeland, India, where my curiosity for science first began.

“The universe is the creation of the mind. The life-force pervades all beings.”

— Rig Veda

"The important thing is not to stop questioning. Curiosity has its own reason for existing."

— Albert Einstein

"To know that we know what we know, and to know that we do not know what we do not know, that is true knowledge."

— Nicolaus Copernicus