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Comparative genomic analysis of necrophagous and parasitic subspecies of *Lucilia cuprina* (Diptera: Calliphoridae) provides important insight into their divergent biologies

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ABSTRACT

Lucilia cuprina, a species of blowfly, consists of two recognized subspecies: *L. cuprina cuprina* and *L. cuprina dorsalis*. Although they are morphologically and molecularly similar to each other, they have very different ecological roles. In Australia, *L. c. dorsalis* is predominantly found in rural areas and is the primary causative agent of sheep myiasis (flystrike), while *L. c. cuprina* is necrophagous and not a significant pest of livestock in the Americas or elsewhere. Here, we present a chromosome-scale genome assembly for *L. c. cuprina* and an improved assembly for *L. c. dorsalis*, enabling comparative genomic analysis between these subspecies. While both genomes share a similar gene content, subspecies-specific genes were identified, which may contribute to their divergent ecological roles —necrophagy in *L. c. cuprina* and parasitism in *L. c. dorsalis*. Phylogenetic analyses across target genomic regions reaffirm the close relationship between *L. c. cuprina* and *L. c. dorsalis* and position *L. sericata* as their sister species. Gene mutations linked to diazinon resistance were exclusively observed in *L. c. dorsalis*, whereas malathion resistance was detected in both subspecies. Additionally, we identified genes with accelerated evolutionary rates in each subspecies, which may underlie their distinct feeding behaviours. We also conducted a detailed analysis of chemosensory genes, revealing that *L. c. dorsalis* possesses slightly larger repertoires of all four chemosensory gene families studied. In comparison to *Drosophila melanogaster*, both subspecies exhibit an expanded gustatory receptor clade. Our findings provide valuable insights into the genetic factors underpinning parasitism and insecticide resistance and provide a valuable genetic resource for future research endeavours, including the development of engineered strains aimed at genetic biocontrol strategies. This work enhances our understanding of the evolutionary adaptations for this important blowfly species.

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1. Introduction

Flystrike, or cutaneous myiasis, is a disease caused by blowfly larvae that is a serious problem in sheep-farming nations, particularly in the United Kingdom, South Africa, the USA, New Zealand, and Australia (Heath et al., 1991; Bisdorff and Wall, 2008; Williams and Villet, 2014; Anstead et al., 2017). Adult female blowflies are attracted to odours associated with damp, soiled fleece or skin of their sheep host (Ashworth and Wall, 1994). They lay eggs on this host, and upon hatching, the larvae migrate towards the skin surface where they feed on secretions, serum, and blood from inflicted wounds as they develop (Barritt and Birt, 1971; Capinera, 2008). The chemical, enzymatic and mechanical effects of these larvae (maggots) progressively lead to disease (flystrike) and, in severe cases, death of the host (Hall and Wall, 1995; Anstead et al., 2017).

Two closely related blowfly species, *Lucilia cuprina* (Wiedemann) and *Lucilia sericata* (Meigen), are globally distributed and pose an ongoing threat to the livestock industry as they both cause myiasis (Wall et al., 1992; French and Morgan, 1996; Anstead et al., 2017). Waterhouse and Paramonov (1950) identified (Waterhouse and Paramonov, 1950) two subspecies of the Australian sheep blowfly *L. cuprina* - *L. cuprina cuprina* (Wiedemann) and *L. cuprina dorsalis* (Robineau-Desvoidy) which share common morphological characteristics (Fig. 1). *Lucilia c. cuprina* has a wide global distribution and is found in China, Japan, North and South America and Australia (Nelson et al., 2012; Kapoor et al., 2023), whereas *L. c. dorsalis* is found in Africa, India and Australia (Stevens and Wall, 1996; Kapoor et al., 2023, 2024). *Lucilia c. cuprina* and *L. c. dorsalis* are difficult to distinguish without genetic analyses (Stevens and Wall, 1995). Differentiation of these subspecies is further complicated by their potential to form morphologically similar hybrids, such as in the eastern parts of Australia (Gleeson and Heath, 2010). In addition, *L. sericata* can also cohabit and hybridise with *L. c. dorsalis* and with *L. c. cuprina* in the laboratory (Li et al., 2014), further complicating species identification and differentiation (Wrigth, 1978; Nelson et al., 2012; Anstead et al., 2015).

In Australia, *L. c. cuprina* typically inhabits urban areas and is opportunistic, whereas *L. c. dorsalis* is almost exclusively parasitic and inhabits rural farmland (Gleeson and Sarre, 1997; Stevens and Wall, 1997; Nelson et al., 2012). The subspecies *L. c. dorsalis* is the primary cause of flystrike in Australia, accounting for ~90 % of cases, leading to substantial economic losses estimated at $324 million annually in the wool industry, due to increased morbidity, treatment costs, and diminished productivity in meat and wool production (Shephard et al., 2022). In contrast, only the *L. c. cuprina* subspecies is found in the Americas (Waterhouse and Paramonov, 1950). Unlike in Australia and New Zealand, *L. c. cuprina* is not associated with sheep myiasis in North America (Hall and Townsend, 1977). There have been documented human myiasis cases due to *L. c. cuprina* (Sherman, 2000; Ahadizadeh et al., 2015). However, this appears to be a rare occurrence as a survey of human myiasis cases (n = 42) in the USA found that *L. sericata* was the commonest causative agent (71.43% of patients), whereas *L. c. cuprina* was only isolated from one patient (Sherman, 2000). Interestingly, *L. sericata* is predominantly responsible for significant outbreaks of flystrike in sheep populations in northern Europe (Wall et al., 1992; French and Morgan, 1996; Diakova et al., 2018), is known to colonise carrion and/or living hosts in South Africa and the United States (Tourle et al., 2009; DeBry et al., 2010; Williams and Villet, 2014; Ahadizadeh et al., 2015) but is not considered a primary initiator of flystrike in Australia (Wall et al., 1992; French and Morgan, 1996; Diakova et al., 2018).

The control of *L. c. dorsalis* on sheep farms in Australia relies largely on the widespread use of broad-spectrum chemical insecticides and integrated management practices (e.g., mulesing, crutching and shearing). The extensive and often indiscriminate use of insecticides against *Lucilia* spp. has led to widespread resistance across multiple classes (Sales, 2020; Sales et al., 2020). In Australia, insecticides (e.g., dicyclanil and cyromazine) are heavily relied upon, alone or in combination with surgical mulesing (breech modification), for flystrike control in high-risk sheep farming regions (Anstead et al., 2017). However, recent reports of concurrent insecticide resistance to these chemicals that have previously offered long-term intervention indicate that resistance management plans need to be reassessed to prevent the loss of field efficacy of current insecticides, given that there are very few alternatives (Sales et al., 2020). In addition, the sheep meat and wool industries aim to reduce the reliance on mulesing to enhance animal welfare outcomes (Anstead et al., 2017). Therefore, insights into the genetic diversity within *L. cuprina* will be/are critical to understand insecticide resistance.

Efforts to investigate genetic diversity, population structure, and track drug resistance require high-quality genomic resources, which are currently lacking for these two subspecies. Here we present, for the first time, a chromosome-scale assembly of the genome of *L. c. cuprina* from the USA and an improved assembly of *L. c. dorsalis* from Australia. We compare the genomes, identify genes associated with insecticide resistance and chemosensation, and those that appear to be under positive selection. We performed phylogenetic analyses using anchored hybrid enrichment sequencing of genomic DNA and with whole blowfly nuclear genomes (n = 15). Overall, our results provide important genomic insight into the distinct biologies of the two subspecies of *L. cuprina* and provide a solid foundation for future functional studies.

2. Material and methods

2.1. Sample collection

The LA07 *L. c. cuprina* strain was obtained from Dr. Aaron Tarone (Texas A&M University) who established the colony from multiple collections of individuals (n = 300–500) from the Miracle Mile neighbourhood (34.0595° N, 118.3491° W) and the University of Southern California campus in Los Angeles, CA, USA. The colony was maintained by using 93 % ground beef as the larval diet and adults were fed water, sugar and protein-rich cookie as described previously (Li et al., 2014; Davis et al., 2018). A highly inbred strain, Lc7/37, was obtained after 10 generations of full sibling single pair matings. For each generation, several (20 or more) single pair matings were set with virgin siblings. The offspring of one of the fertile crosses was randomly selected for collecting virgin flies to set the next generation of crosses.

A laboratory strain (LS) of *L. c. dorsalis* was isolated from the Australian Capital Territory, Australia (35.4735° S, 149.0124° E) and maintained for more than 20 years in the laboratory of Dr Peter J. James (The University of Queensland, Australia) using an established culture method (Greenberg, 1973; Anstead et al., 2017). The strain was later transferred to the laboratory in The University of Melbourne, where it has continued to be maintained as previously described, with the larval diet consisting of wet cat food (Whiskas® Jellymeat Loaf) and adult flies fed with water and Sustagen (Hospital formula, vanilla flavour). Five lines of *L. c. dorsalis* were established under controlled conditions at 28 °C and 80 % relative humidity in separate cages at The University of Melbourne laboratory. These lines were inbred for six generations to minimize genetic variability (Greenberg, 1973). Male offspring from the fertile crosses were collected for subsequent next-generation sequencing.

The GG strain of *L. c. dorsalis* was established from larvae collected from sheep from New South Wales, Australia (35.4926°

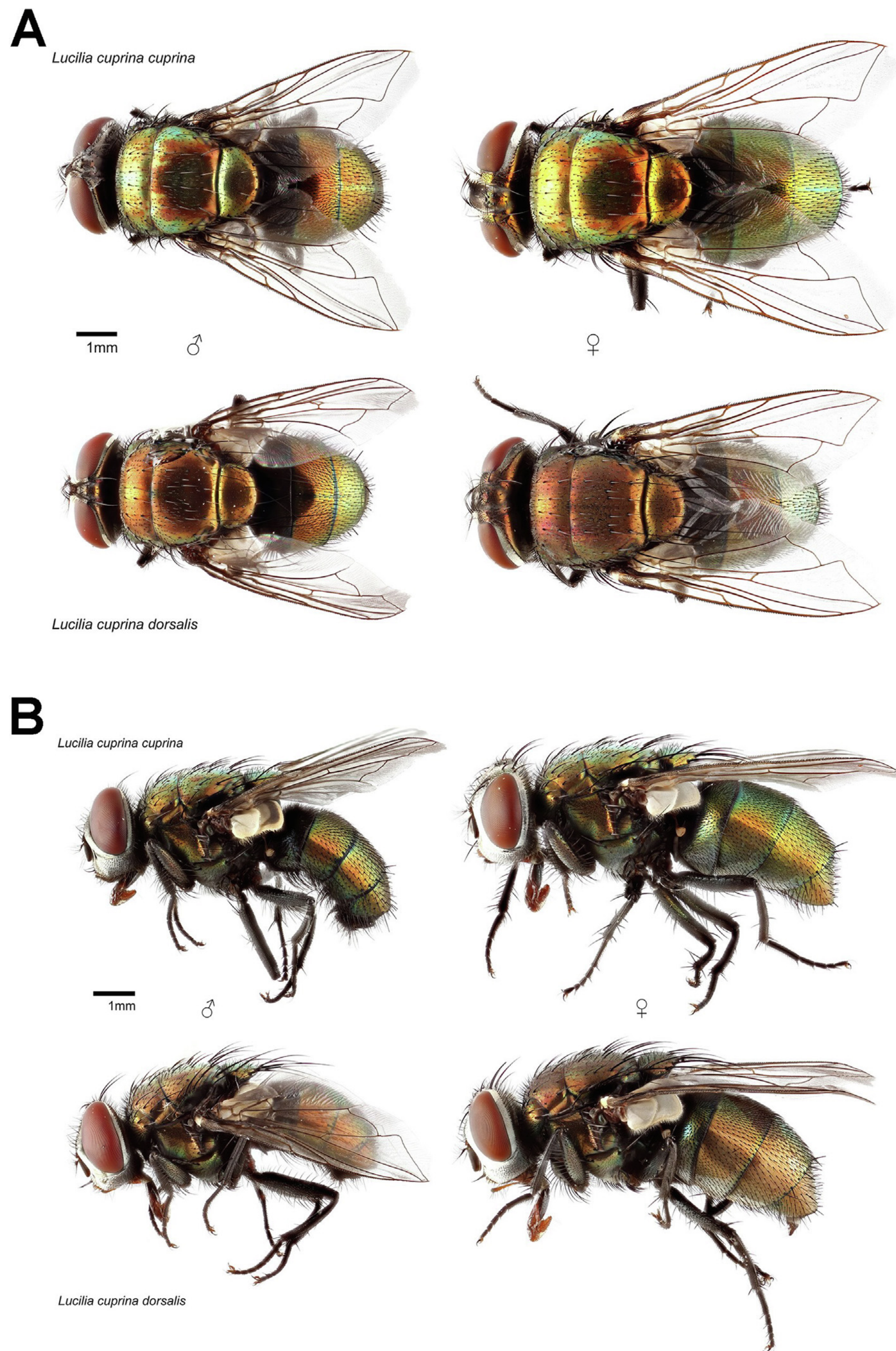

Fig. 1. Morphology of *Lucilia* subspecies. *Lucilia cuprina cuprina* and *Lucilia cuprina dorsalis* have a metallic coppery appearance. A. Top view of *L. c. cuprina* (LA07 strain) and *L. c. dorsalis* (GG strain) male and female. B. Side view of *L. c. cuprina* (LA07 strain) and *L. c. dorsalis* (GG strain) male and female.

S, 147.5457° E), inbred and imported to the USA where it has been maintained in an ACL2 laboratory at NC State University under the same rearing conditions as used for the LA07 strain of *L. c. cuprina*. The adults were collected and stored in 95 % ethanol for imaging purposes only.

2.2. Genomic DNA isolation, library construction and sequencing

For *L. c. cuprin*a, high molecular weight DNA was isolated from embryos from the Lc7/37 line following established procedures (Linger et al., 2015). Library construction and Illumina DNA sequencing (150 bp paired end) was performed by Dovetail Genomics (now Cantata Bio, Scotts Valley, CA., USA). For Hi-C sequencing, libraries were prepared from late-stage Lc7/37 embryos (17–18 h at 21 °C) and were collected and shipped frozen to Dovetail Genomics. For HiFi PacBio sequencing, high molecular weight DNA was extracted from a single Lc7/37 adult male (Linger et al., 2015). Library preparation and PacBio DNA sequencing were done by the Georgia Genomics and Bioinformatics Core with a Sequel II machine (Athens, GA., USA) (Fig. 2), resulting in > 50 Gb of data.

However, only about 1 Gb was HiFi and so continuous long reads (CLR) were used for this sequencing data. The subread statistics were calculated using the SequelTools QC tool (Hufnagel et al., 2020) and the number of subreads was 9,692, 445. The mean subread length was 5,526 bp and the subread N50 was 6,698 bp.

For *L. c. dorsalis*, high molecular weight DNA was isolated from adult males of LS strain using the previously established method using Phenol-chloroform extractions (Green and Sambrook, 2017). Illumina DNA sequencing (150 bp paired end) was performed by Dovetail Genomics Production Lab (Santa Cruz, CA, USA). Library preparation and PacBio DNA sequencing were done using two SMRT cells on PacBio RS II machine by Macrogen Inc. (Korea) (Fig. 2). The Dovetail and PacBio sequencing yielded > 50 Gb and 38 Gb of data, respectively, with the majority of the PacBio data consisting of continuous long reads (CLR).

2.3. Nuclear genome assembly, annotation and comparison

For *L. c. cuprina*, the Illumina short reads (150 bp PE) were assembled with Meraculous (Chapman et al., 2011) by Dovetail Genomics. Chicago (150 bp PE) and Hi-C libraries (Dovetail HiRise) from Dovetail Genomics were used for scaffolding. PacBio CLR were used to gap fill the scaffolded assembly using TGS-GapCloser (v1.1.1) (Xu et al., 2020) with default settings. Prior to this step, the reads were error corrected and trimmed using Canu (v2.1.1) (Koren et al., 2017). To reduce the read depth for gap filling, BBTools (https://jgi.doe.gov/data-and-tools/software-tools/bbtools/) was used to partition the 2,407,770 reads into three equally sized sets. One read set was selected for use in gap filling and BBTools was used to remove any SMRTbell adapters in the read set, resulting in 802,223 reads. After gap filling with TGS-GapCloser, Pilon (v1.24) (Walker et al., 2014) was used to polish the single nucleotide polymorphisms (SNPs) and Indels. This polish step used the Illumina short reads as input, where the reads were initially quality filtered using fastp (v0.21.0) with the -q 30 parameter. The resulting reads were subsampled to 175×10^6 pairs using BBTools and then mapped to the gap-filled assembly using Bowtie2 (v2.4.4) (Langmead and Salzberg, 2012). Four scaffolds were removed that were either potential contaminants or mis-assemblies. The genome was annotated by NCBI using the NCBI Eukaryotic Genome Annotation Pipeline with previously published RNA-seq data (Davis et al., 2018; Williamson et al., 2021) and from Bioproject PRJNA275749 (see Fig. 2). The Bioprojects associated with the genome assembly are PRJNA809378 and PRJNA787202.

For *L. c. dorsalis*, Illumina short reads (150 bp PE) generated by Dovetail Genomics Chicago were assembled with Dovetail HiRise (v2017-01-10) (Pruitt et al., 2014). PbJelly2 (vPBSuite15.8) (English et al., 2012) was used to close the gaps in the draft assembly using long reads generated from PacBio sequencing. The Illumina short reads were used to polish the assembly with Pilon (v1.21) (Walker et al., 2014). The pre-assembled contigs were scaffolded using SSPACE-LongRead (v1.1) (Boetzer et al., 2011). PbJelly2 (vPBSuite15.8) (English et al., 2012) was again used to close the gaps in the draft assembly. HaploMerger2 (v3.2) (Huang

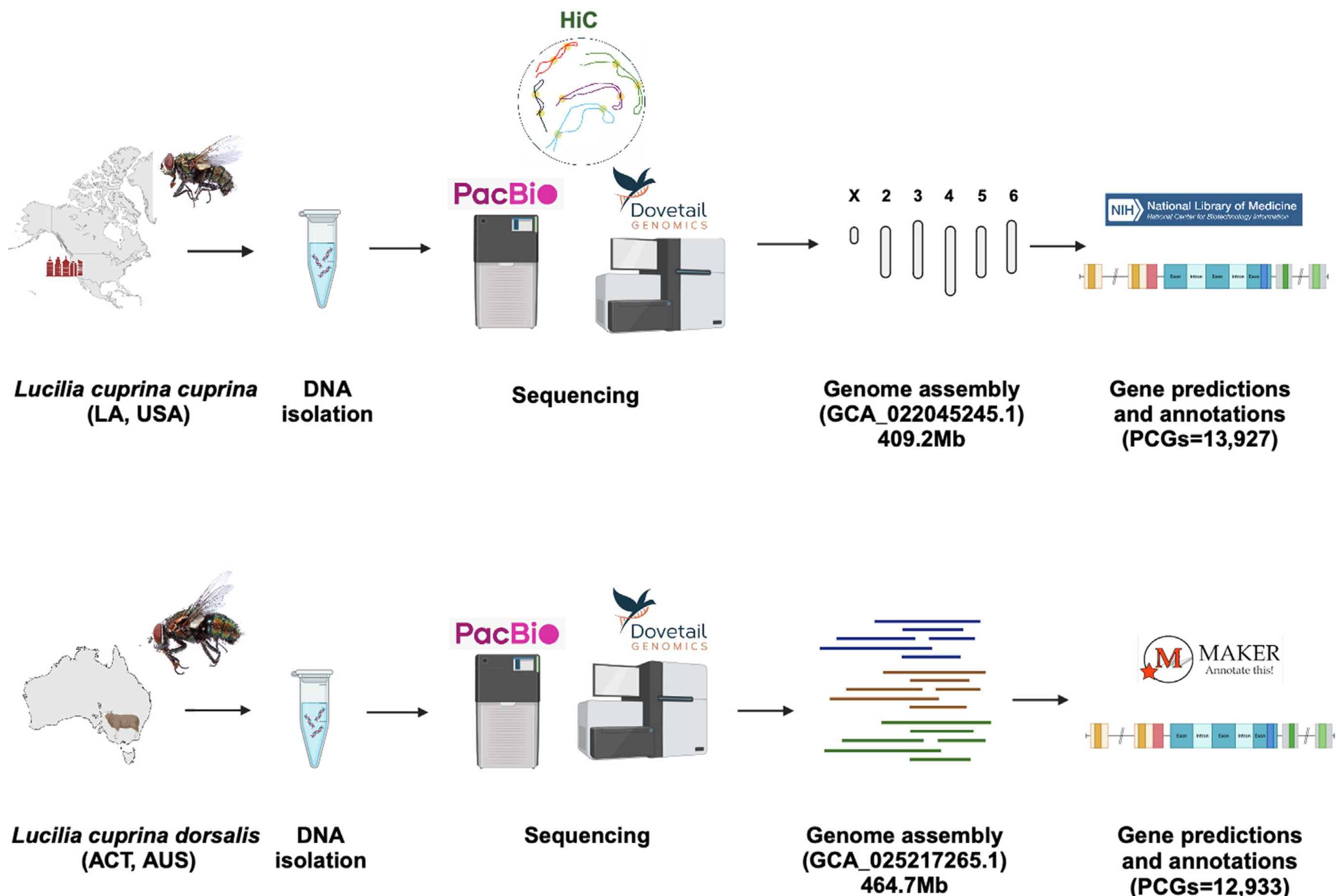

Fig. 2. An overview of assembly methods. The workflow used for the *Lucilia cuprina cuprina* and *Lucilia cuprina dorsalis* genome assemblies and annotation are shown.

et al., 2017) was used for improving the draft genome assembly in the post-assembly stage. The MAKER genome annotation pipeline based on RNA-seq data and protein homology was used to annotate the genome assembly (Cantarel et al., 2008; Campbell et al., 2014) (see Fig. 2). K-mer based estimates of heterozygosity was obtained using Meraculous (Chapman et al., 2011) for *L. c. cuprina* and GenomeScope 2.0 (Ranallo-Benavidez et al., 2020) for *L. c. dorsalis*.

Genomic feature statistics were collated for both the genome assemblies (GenBank *L. c. cuprina*: GCA_022045245.1 and *L. c. dorsalis*: GCA_025217265.1) and GSAlign (Lin and Hsu, 2020) with default settings was employed to align the two genomes and identify variations, including SNPs, insertions, and deletions between the two genomes. OrthoFinder (v2.5.5) (Emms and Kelly, 2019) was used to identify orthologous genes between the genomes of the two *Lucilia* subspecies. An orthogroup was defined as a set of genes that have descended from a single gene from the last common ancestor (LCA) in a species clade (Emms and Kelly, 2019). Protein sequences from the two subspecies - *L. c. cuprina* (GCA_022045245.1) and *L. c. dorsalis* (GCA_025217265.1) were used as input for OrthoFinder, which was run with default settings to predict orthogroups and species-specific genes. The overall synteny between the *L. c. cuprina* and *Drosophila melanogaster* genomes was examined using orthogroups as described previously for the *Cochliomyia hominivorax* genome (Tandonnet et al., 2023).

2.4. Insecticide resistance mutation analyses

Analysis of resistance alleles to targets of current or historical commercial insecticide chemistries used in treatment and protection against flystrike was performed. For each insecticide target, the amino acid sequences were retrieved from the NCBI database for *L. c. cuprina* and *L. c. dorsalis* and aligned using the MUSCLE aligner (v5.1) (Edgar, 2004) in Geneious Prime® (v2019.2.3) software (Kearse et al., 2012). The alignments were checked for specific resistance mutations that had been previously documented (Supplementary Table 1).

2.5. Phylogenetic analyses

2.5.1. Taxon sampling

Our genomic dataset (n = 15 taxa) included 14 Calliphoridae species and a single outgroup taxon from Tachinidae, *Tachina fera* (Linnaeus). Unless noted, all genome assemblies were publicly available and downloaded from NCBI (Supplementary Table 2).

Our anchored hybrid enrichment (AHE) dataset (n = 17 taxa) included 13 *Lucilia* species/subspecies and one outgroup taxon from subfamily Chrysomyinae, *C. hominivorax* (Coquerel). Specimens were collected and identified by colleagues and project collaborators (Supplementary Table 2). All specimens were collected in compliance with regulations and permits from the country of origin and transferred to the NC State University Insect Collection under CITES permit #08US827653/9.

2.5.2. Anchored hybrid enrichment sequencing

Whole genomic DNA (gDNA) was extracted using the DNeasy Blood and Tissue Kit (Qiagen) from whole flies included in Fig. 5 using thoracic and leg tissue following the manufacturer's recommendations. The DNA from each sample was quantified using a Qubit fluorometer (High Sensitivity Kit, Life Technologies, Inc.) and sheared to a target size of approximately 300 bp in Covaris tubes (Covaris, Inc.) using sonication. Sheared DNA was used for gDNA library preparation with a modification of the protocol (Meyer and Kircher, 2010). A size selection step was added using SPRIselect beads at a 0.9x ratio of bead to sample volume (Beckman Coulter, Inc.). Indexes were added to the samples and assembled into pools of 24 samples per pool. Each pool was enriched using the Diptera AHE kit (Young et al., 2016) using an Agilent Custom SureSelect kit which targets 559 curated loci using tiled, custom probes (Agilent Technologies). Loci were designed by comparison across seven genomes and 14 transcriptomes of Diptera species provided by the 1KITE consortium. All targeted loci were 150 bp or longer. Paired read sequencing was performed using Illumina technology with 150 bp paired end reads (NC State University Genomic Sciences Laboratory).

2.5.3. Data processing and phylogenetic analysis

Raw reads were first demultiplexed at the NC State University Genomic Sciences Laboratory using cassava (v1.8.2) (Institut Pasteur), then adapters and poor-quality sequences were trimmed using fastp (v0.2048) (Chen et al., 2018) for NovaSeq sequenced samples or using trimmomatic (v0.3647) (Bolger et al., 2014) for MiniSeq sequenced samples. Sequences were eliminated from the sample if they were detected as belonging to organisms outside of Diptera through BLASTN (NCBI) using an E-value threshold of 1e-5. These 'cleaned' reads were then assembled using trinity 2.4 (Grabherr et al., 2011) or SPAdes (Bankevich et al., 2012). Assembled reads then underwent orthology assessment by single-copy ortholog calling and hmm graphical mapping, using the default parameters in orthograph (v0.5.14) (Petersen et al., 2017), except for "soft-threshold = 0" and "max reciprocal-mismatches = 0". Gene models for captured loci were identified within a curated set of single copy orthologs, the established BrachyBase ortholog set, based on seven schizophoran Diptera species in the OrthoDB database (Winkler et al., 2022). Assembled sequences were aligned with mafft (v7.273) (Katoh and Standley, 2013) using the L-INS-I algorithm. To obtain nucleotide versions of each amino acid multiple sequence alignment (MSA), a modified version of pal2nal (v0.14) (Suyama et al., 2006) was used with modifications as described by Misof et al. (Misof et al., 2014). Maximum Likelihood (ML) phylogenetic trees were calculated on our datasets of both the nucleotide and amino acid MSAs using IQTree (v1.6.8) (Nguyen et al., 2015) to infer models for each gene. ML analyses were performed using default parameters with 1000 ultrafast bootstrap replicates and 1000 SH-aLRT test replicates. In the data set using only AHE harvested sequences, a total of 2,639 loci were concatenated in a single unpartitioned phylogenetic data set for ML analysis. For the whole genome analysis, a total of 5,402 unpartitioned loci were included in the final concatenated data set. All analyses were performed on the NC State University Bioinformatics Research Center's High-Performance Computing Cluster.

2.6. Positive selection analyses

A total of 5,402 inferred single copy orthologs from the whole genome analysis phylogenetic tree for *L. c. cuprina* and *L. c. dorsalis* coding sequences were analyzed to calculate the rates of molecular evolution using an established pipeline (Scott et al., 2020). Orthologs were realigned and cleaned using TranslatorX (Abascal et al., 2010). The alignments were then used as input for evolutionary model testing in CodeML within the PAML 4 software (Yang, 2007), where we estimated rates of synonymous (d_S) and non-synonymous (d_N) substitution rates and their ratio (ω or d_N/ d_S).

Two clusters of orthologous genes were designated: one for *L. c. cuprina* (5,342 orthologs) and another for *L. c. dorsalis* (5,234 orthologs), with each cluster assigning the focal subspecies as foreground and the rest of the tree as background. We tested three models: (a) a *M0* model with a fixed ω = 1 (null hypothesis), (b) a *b_neut* model with $\omega_{foreground}$ fixed at 1 and $\omega_{background}$ free to vary (model 1), and (c) a *b_free* with $\omega_{foreground}$ and $\omega_{background}$ free to vary (model 2). A likelihood ratio test (LRT) compared models 1

and 2 against the null hypothesis using a χ^2 test with a significance threshold set at 0.05, and the *p* values adjusted using the false discovery rate (FDR) method. Results were interpreted to identify genes under positive or purifying selection based on the $\omega_{foreground}$ ratio, with $\omega_{foreground} > 1$ indicating positive selection and $\omega_{foreground} < 1$ indicating purifying selection. Genes showing selective shifts exclusive to each subspecies were considered candidates for roles in parasitic or necrophagous adaptations. Genes with $\omega_{foreground}$ higher than the $\omega_{background}$ were inferred as having accelerated evolutionary rates, while those with $\omega_{foreground}$ lower than the $\omega_{background}$ were considered as having decelerated evolutionary rates.

We conducted a Gene Ontology (GO) enrichment analysis on genes with different evolutionary rates using TopGO in R (Alexa and Rahnenfuhrer, 2024) using Fisher exact test, with the complete set of 5,402 orthologs as background. We compared median molecular evolution rates among GO terms (across the three categories) and the genome median to identify processes or functions with significant deviations. To achieve this, we filtered the GO terms to remove redundancy using REVIGO (Supek et al., 2011) and retained only those with at least 30 associated genes. The median ω for each GO term was calculated and compared to the genomic median using a Mann-Whitney test implemented in R to identify GO terms with median ω values significantly different from the genomic median and corrected the results using FDR method.

2.7. Chemosensory gene families

Manual annotation of odorant receptors (ORs), gustatory receptors (GRs), ionotropic receptors (IRs), and odorant binding proteins (OBPs) was conducted by BLASTn and tBLASTn analyses of both *L. cuprina* genome assemblies using *Cochliomyia hominivorax* gene models as queries (Scott et al. 2020). BLAST analyses were completed using Geneious Prime® (v2023.2.1) (https://www.geneious.com). Intron donors and acceptors, when ambiguous, were assessed using the Splice Site Prediction by Neural Network software (Reese et al., 1997) on the Berkeley Drosophila Genome Project website (https://www.fruitfly.org/seq_tools/splice.html). The *L. cuprina* gene models were evaluated with the aid of multiple protein alignments and gene trees containing the *C. hominivorax* chemosensory proteins. When possible, gene models with indels or premature stop codons were repaired to improve estimation of their phylogenetic relationships. It was apparent that some of these were pseudogenes (i.e., a segment of DNA that structurally resembles a gene but is not capable of coding for a protein) due to the degree and nature of their degradation; however, several contained indels but were otherwise highly conserved. We considered these sequencing errors and not pseudogenes, thus they were labelled "repaired" in Supplementary File 1.

The ORs and OBPs were numbered based on their order in the *L. c. cuprina* genome assembly from chromosome 2–6. The GRs were numbered starting with the putative CO2 receptors (Gr1 and Gr2) followed by the sugar receptors (Gr3–Gr9), with the remaining numbered by their order along the chromosomes. The IRs were named based on homology with *D. melanogaster* IRs, with the divergent IRs being numbered by their order along the chromosomes starting with Ir101. Apparent duplications were suffixed with a hyphen and number, while putative splice variants were indicated with a capital letter (A, B, C, etc.).

Drosophila melanogaster (Meigen) gene models (Hekmat-Scafe et al., 2002; Robertson et al., 2003; Benton et al., 2009) were used for comparative analysis of the *L. cuprina* chemosensory genes. Multiple protein alignments for phylogenetic estimation were conducted using the G-INS-I method in MAFFT (v7) (Katoh et al., 2019). Alignments were trimmed using the strict method in TrimAL (v1.3) (Capella-Gutiérrez et al., 2009) on the Phylemon 2.0 web browser (Sánchez et al., 2011). Phylogenetic relationships among the chemosensory proteins were estimated using IQ-TREE (http://iqtree.cibiv.univie.ac.at/) with the auto substitution option and 1000 ultrafast bootstrap replications (Trifinopoulos et al., 2016). Figures were produced using the interactive Tree of Life (iTOL) (v6.8.2) software (https://itol.embl.de/). The ORs were rooted at the odorant co-receptor (Orco), the GRs were rooted at the clade containing the CO2 receptors (Gr1 and Gr2), the IRs were rooted at the Ir25a/Ir8a clade, and OBPs were rooted at the midpoint.

3. Results

3.1. Genome organisation, composition and comparison

The genome size for *L. c. cuprina* and *L. c. dorsalis* was 409.2 Mb (total number of chromosomes, scaffolds and contigs: 6; 8,457; 27,983) and 464.7 Mb (total number of chromosomes, scaffolds and contigs: 0, 560; 32,993), respectively (Table 1). The heterozygosity estimated from k-mer analysis was relatively low for both subspecies, with *L. c. cuprina* at 0.08 % and *L. c. dorsalis* at 0.59 %, indicating limited residual genetic variation. The GC content for each genome assembly was 29.5 %. In *L. c. cuprina*, the scaffold and contig N50 lengths were 71 Mb and 65.3 kb, respectively, while in *L. c. dorsalis*, the corresponding lengths were 6.9 Mb and 33.8 kb. The genome completeness, as measured by the Benchmarking Universal Single-Copy Orthologs (BUSCO) score (diptera_odb10), was 97.1 % for *L. c. cuprina* and 97.2 % for *L. c. dorsalis* (Table 1). The proportion of single-copy and duplicated BUSCOs for *L. c. cuprina* was 96.3 % and 0.8 %, respectively, whereas for *L. c. dorsalis*, these values were 96.7 % and 0.5 %, respectively. The total number of protein-coding genes (PCGs) was 13,927 for *L. c. cuprina* and 12,933 for *L. c. dorsalis*. The number of SNPs, insertions and deletions between the two *Lucilia* subspecies was 5,069,614, 694,407 and 645,415, respectively (Table 2).

The OrthoFinder analysis identified 29,483 orthogroups among the two *L. cuprina* subspecies including a total of 6,865 single-copy orthologous genes (Table 3). Among these, 10,284 orthogroups were shared between *L. c. cuprina* and *L. c. dorsalis*. Additionally, 543 subspecies-specific orthogroups were identified, containing 1,934 subspecies-specific genes, some of which were novel in these subspecies (Table 3). Notably, *L. c. dorsalis* exhibited a higher number of subspecies-specific orthogroups compared to *L. c. cuprina*, with 6.9 % of its genes assigned to these groups, whereas only 5.1 % of the genes in *L. c. cuprina* were assigned to subspecies-specific orthogroups (Table 4). In *L. c. dorsalis*, 598 out of 1,129 subspecies-specific genes encoded hypothetical or uncharacterised proteins, while in *L. c. cuprina*, 328 out of 790 subspecies-specific genes were similarly associated with hypothetical or uncharacterised proteins (Supplementary File 2).

The extensive chromosome linkage group maps for *L. c. dorsalis* (Weller and Foster, 1993) were used to assign the longest scaffolds of the *L. c. cuprina* assembly to the numbered chromosomes. Many of the phenotypic mutations that were mapped genetically such as "purple body" and "balloon wings" could not be unambiguously assigned to genes on the scaffolds. However, for other phenotypic or insecticide resistant mutations, the molecular basis is known. For example, the eye color mutants *white*, *yellowish*, *topaz* and *yellow* are the *Lucilia* homologs of the *Drosophila white*, *vermilion*, *scarlet* and *cinnabar* genes, respectively (Summers and Howells, 1978; Elizur et al., 1990), and the *Scalloped wings* gene is the ortholog of *Notch* (Chen et al., 1998). The loci corresponding to these genes were identified in the *L. c. cuprina* assembly (Fig. 3). We also included genes where the corresponding *Drosophila* ortholog was

Table 1
A comparison of genome features of *Lucilia* subspecies *(Lucilia cuprina dorsalis* and *Lucilia cuprina cuprina).*

Description	*Lucilia cuprina dorsalis*	*Lucilia cuprina cuprina*
Assembly level	Scaffold	Chromosome
Genome size (Mb)	464.7	409.2
Total ungapped length (Mb)	439.7	407.2
Total number of chromosomes	-	6
Total number of scaffolds; contigs	560; 32,993	8,457; 27,983
Scaffold N50 length in Mb	6.9	71
Scaffold L50	19	3
Contig N50 length in kb	33.8	65.3
Contig L50	2,981	1,498
GC content of the whole genome (%)	29.5	29.5
Repetitive sequences (%)	22.59	54.29
Total number of genes	13,009	15,856
Total number of protein-coding genes	12,933	13,927
Gene size (mean; bp)	16110.6	13048.5
CDS size (mean; bp)	341.1	392.8
Genome completeness (%)	97.2	97.1
Single-copy orthologues genes	11,142	13,412
GenBank No.	GCA_025217265.1	GCF_022045245.1

Table 2
Number of single nucleotide polymorphisms (SNPs), insertions and deletions between *Lucilia* subspecies *(Lucilia cuprina dorsalis* and *Lucilia cuprina cuprina).*

	*Lucilia cuprina dorsalis*SNPs, insertions, deletions	*Lucilia cuprina cuprina*SNPs, insertions, deletions
Lucilia cuprina dorsalis	0, 0, 0	5069614, 694407, 645415
Lucilia cuprina cuprina	5069614, 694407, 645415	0, 0, 0

Table 3
Comparative statistics of the two *Lucilia* subspecies (*Lucilia cuprina cuprina* and *Lucilia cuprina dorsalis*) from the OrthoFinder analysis.

Number of species	2
Number of protein coding genes	32151
Number of genes in orthogroups	29483
Number of unassigned genes	2668
Percentage of genes in orthogroups	91.7
Percentage of unassigned genes	8.3
Number of orthogroups	10827
Number of species-specific orthogroups	543
Number of genes in species-specific orthogroups	1934
Percentage of genes in species-specific orthogroups	6
Mean orthogroup size	2.7
Median orthogroup size	2
G50 (assigned genes)	3
G50 (all genes)	2
O50 (assigned genes)	3529
O50 (all genes)	4124
Number of orthogroups with all species present	10284
Number of single-copy orthogroups	6865

identified on the basis that the *Drosophila* and *Lucilia* mutations had very similar phenotypes and were in the same linkage group (Weller and Foster, 1993), but had not yet been confirmed at the molecular level. These include the *Lucilia aristapedia*, *no steady state* and *eyeless* genes that were the potential orthologs of the *Drosophila spineless*, *transient receptor potential* and *eyes absent* genes, respectively. The recombination map distances between the genes determined by Foster and colleagues (Weller and Foster, 1993) are shown on the map. Although the recombination mapping was done with *L. c. dorsalis*, there is a correlation between the map distances and the physical separation of the genes on a scaffold. The order of genes on chromosomes 3 and 4 were the same as the recombination map except for the *nss* gene, which is upstream of *Rop* on the scaffold but to the right of *Sh* on the linkage map. Genes placed at the beginning of a chromosome by recombination mapping (*Rdl* on chromosome 5 and *vg* on chromosome 6) were close to the end of a scaffold. Overall, there was a clear correspondence between the chromosomal elements of *D. melanogaster* and the *L. c. cuprina* major scaffolds (Supplementary Fig. 1). The smallest of the six major scaffolds corresponded to the X chromosome, which largely contains the orthologs of *D. melanogaster* fourth chromosome genes as previously reported (Linger et al., 2015; Davis et al., 2018).

Table 4
Orthologous protein clustering statistics for *Lucilia cuprina cuprina* and *Lucilia cuprina dorsalis.*

	L. c. cuprina	*L. c. dorsalis*
Number of protein-coding genes	15761	16390
Genes in orthogroups (%)	91.4	92
Orthogroups containing species (%)	97.1	97.9
Number of species-specific orthogroups	229	314
Number of genes in species-specific orthogroups	790	1129
Genes in species-specific orthogroups (%)	5.1	6.9

The full species-specific clustering summary can be found in the Supplementary File 2.

3.2. Insecticide resistance alleles in Lucilia cuprina cuprina and Lucilia cuprina dorsalis

The presence of known resistance alleles to targets of current or historical commercial insecticide chemistries used in treatment and protection against flystrike was examined. For both *L. c. cuprina* and *L. c. dorsalis,* our analysis did not show any resistance alleles to compounds like spinosyns, neonicotinoids, macrocyclic lactones, or pyrethroids (Supplementary Table 1). However, insecticide-resistant alleles were identified in the gene encoding the a7-esterase enzyme, which is associated with resistance to diazinon (*Rop-1*) or malathion (*Rmal*), depending on the specific resistance allele detected.

For esterase B1 (*Rop-1*), the resistant allele Aspartic acid (137) is present in *L. c. dorsalis* (GenBank Accession Number: KAI8129144), while the susceptible allele Glycine (137) is in *L. c. cuprina* (GenBank Accession Number: XP_023292034.2). Further potential sites associated with resistance were examined including Alanine (267) in *L. c. cuprina*, where the substitution to Valine in *L. c. dorsalis* is associated with resistance. Methionine (283) in *L. c. cuprina* (susceptible) is another site associated with resistance when substituted with Leucine (283) in *L. c. dorsalis*. Threonine (335) and Isoleucine (358) in *L. c. cuprina* are associated with susceptibility while Histidine (335) and Phenylalanine (358) are associated with resistance in *L. c. dorsalis* (Supplementary File 3). For *Rmal* gene (GenBank Accession Number: KAI8129144), the substitution of Tryptophan (251) (susceptible allele) to Leucine (251) (resistant allele) was noted. The resistant allele is present in *L. c. cuprina* (GenBank Accession Number: XP_023292034.2) (Supplementary File 3).

3.3. Phylogenetic relationships

We present a broadly sampled phylogeny of Calliphoridae representatives using 5,402 loci from whole nuclear genomic data (Fig. 4) as well as a focused phylogeny of relevant *Lucilia* representatives using 2,639 loci harvested from anchored hybrid enrichment (AHE data) (Fig. 5). We found *L. cuprina* subspecies *L. c.*

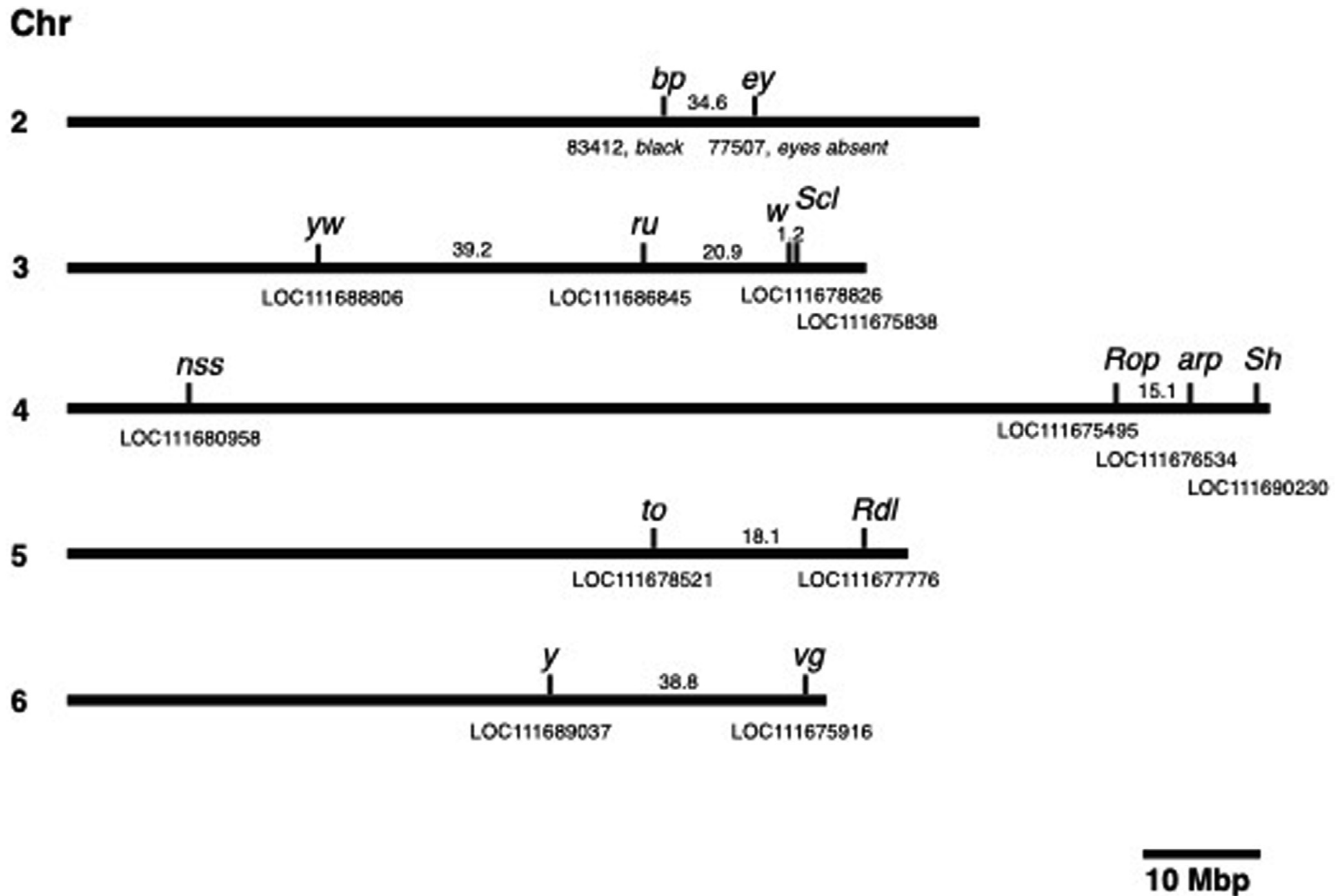

Fig. 3. Location of genes on *L. c. cuprina* scaffolds that were previously genetically mapped to chromosomal linkage groups. The scaffold lengths, gene locations and recombination map distances between genes determined by Weller and Foster (Weller and Foster, 1993) are shown. Gene abbreviations: *bp*, black puparium; *ey*, eyeless; *yw*, yellowish eyes; *ru*, rusty body; *w*, white eyes; *Scl*; Scalloped wings; *nss*, no steady state; *Rop1*, Diazinon resistance; *arp*; aristapedia; *Sh*, short bristles; *to*, topaz eyes; *Rdl*, Dieldrin resistance; *y*, yellow eyes; *vg*, vestigial wings.

cuprina and *L. c. dorsalis* correctly placed to genus, with all *L. c. cuprina* individuals monophyletic, as expected. We also recovered *L. sericata* as sister to *L. cuprina* and Luciliinae as sister to Calliphorinae. All nodes had absolute support.

3.4. Positive selection

To identify genes potentially involved in the evolutionary divergence of *L. c. cuprina* (primarily necrophagous) and *L. c. dorsalis* (primarily parasitic) lifestyles, we examined shifts in selective regimens across branches, focusing on each subspecies separately. Our objective was to discover genes under differential selective pressures in both subspecies, potentially linked to the parasitic lifestyle.

We identified 315 genes with a best fit with the *b_free* model ($\omega_{foreground}$ and $\omega_{background}$ free to vary) with *L. c. cuprina* as the focus subspecies (Supplementary Table 3), and 325 genes using *L. c. dorsalis* as the focus subspecies (Supplementary Table 4). Among these, we observed a similar number of genes with accelerated evolutionary rates in each subspecies (i.e., 245 in *L. c. cuprina* and 237 in *L. c. dorsalis*), and a similar number of genes with decelerated evolutionary rates (i.e., 49 in *L. c. cuprina* and 51 in *L. c. dorsalis*).

Using the 315 genes with a shift in the evolutionary rate, we conducted an enrichment analysis across the three gene ontology categories: Biological Process (BP), Cellular Component (CC), and Molecular Function (MF), for both subspecies. In *L. c. cuprina*, 30 GO terms were associated with genes showing accelerated evolutionary rates (16 BP, 8 CC, and 6 MF), while 40 were associated with genes with lower rates (24 BP, 8 CC, and 8 MF). In *L. c. dorsalis*, we found 47 GO terms linked to genes with accelerated rates (19 BP, 3 CC, and 25 MF) and 21 associated to genes with lower rates (17 BP, 2 CC, and 2 MF) (Supplementary Figs. 2 and 3). Most GO terms among genes with accelerated rates in both subspecies were primarily linked to catabolic or metabolic processes (Supplementary Fig. 2). In contrast, genes with lower rates in *L. c. cuprina* were primarily associated with transport, while those in *L. c. dorsalis* were mostly linked to muscle or system development (Supplementary Fig. 3).

To further explore the relationship between evolutionary rates and gene ontology, we compared the distribution of ω values across more than 1000 GO terms, by calculating the median ω for each term and comparing them to the calculated genome-wide ω median of 0.073. For example, terms related to "pigmentation" and "extrinsic components of membranes" deviated from the genome-wide ω median in *L. c. cuprina*, while "carbohydrate derivative binding" and "phenol-containing compound metabolic process" deviated in *L. c. dorsalis*. However, after adjusting for multiple comparisons using the FDR method, some GO terms showed deviations in ω values, but none remained statistically significant after correction. We refined the analysis by focusing on the top 30 GO terms with median values with the highest deviations from the genome-wide median, selecting 15 above and 15 below the genome-wide median (Supplementary Figs. 4 and 5).

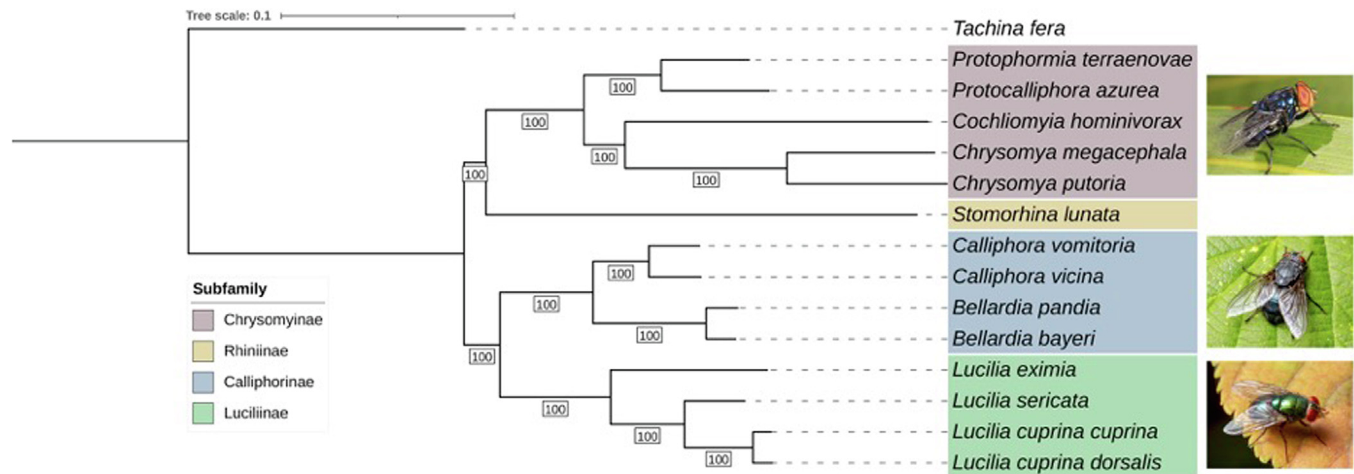

Fig. 4. Maximum likelihood (ML) tree with 1000 ultrafast bootstrap replicates and 1000 SH-aLRT test replicates using nucleotide data from whole genomic sequences. A total of 5402 unpartitioned loci were included in the final data set. Ingroup taxa are coded by subfamily.

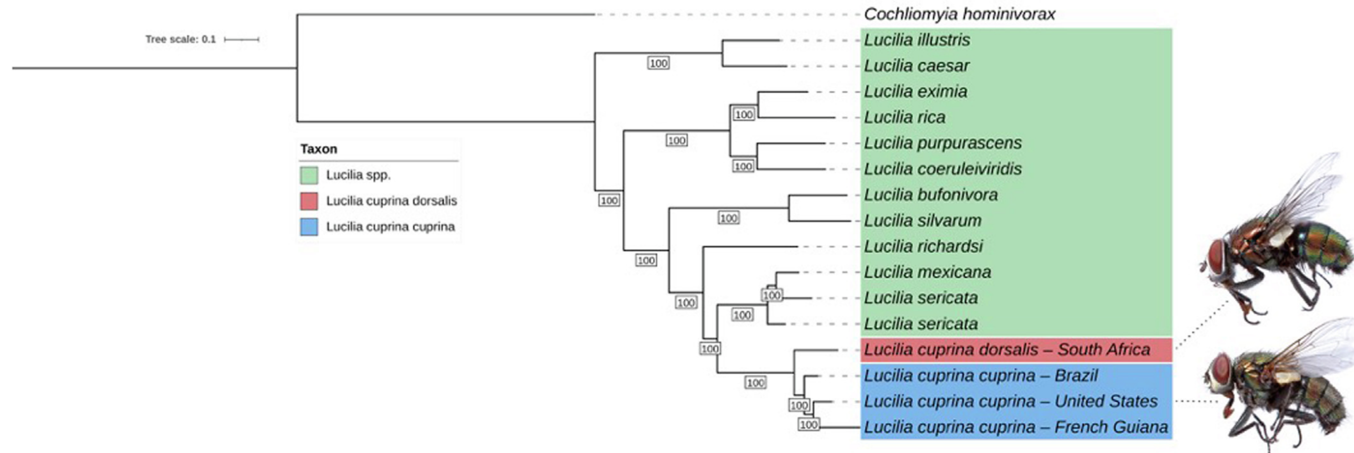

Fig. 5. Maximum likelihood (ML) tree with 1000 ultrafast bootstrap replicates and 1000 SH-aLRT test replicates using nucleotide data harvested from anchored hybrid enrichment sequencing. A total of 2639 unpartitioned loci were included in the final data set. Ingroup taxa are coded by species/subspecies.

3.5. Chemosensory gene families

The chemosensory gene families are evolutionarily dynamic via the birth-and-death model of evolution exemplified by gene duplication, diversification, and loss through pseudogenization (Sánchez-Gracia et al., 2009; Benton, 2015; Robertson, 2019). These receptors can provide valuable insights into adaptation and species evolution because they interact directly with cues in the environment that drive critical behaviours such as host seeking and mating. For example, the size of the OR and/or GR repertoires has been correlated with the complexity of a species' niche; that is, an insect with a more complex niche often has a greater number of chemosensory receptors (Robertson, 2019).

Insect odorant receptors form a tetrameric complex comprising the odorant receptor co-receptor (Orco) and a ligand-specific (canonical) OR (Del Mármol et al., 2021; Zhao et al., 2024). Each *L. cuprina* genome contained a single copy of *Orco*, while the number of canonical OR genes was higher in *L. c. dorsalis* (76 + 6 pseudogenes) than in *L. c. cuprina* (71 + 7 pseudogenes), with the differences belonging to a closely related clade containing an intact Or37 and three Ors (Or79–Or81) not present in *L. c. cuprina* (Fig. 6). The 10 most highly expressed ORs in *L. c. cuprina* antennae based on Wulff et al. (2024) (Wulff et al., 2024a) are highlighted in Fig. 6.

Gustatory receptors (GRs) can be grouped into several types including sugar, CO_2, bitter, and pheromone receptors (Thorne et al., 2004; Montell, 2009). Although highly expressed in taste pegs on mouthparts and legs, some can be found in other tissues such as the antennae of cyclorrhaphan flies where the CO_2 receptors, DmelGr21a and DmelGr63a, are expressed (Couto et al., 2005; Jones et al., 2007; Kwon et al., 2007). As with the ORs, the total number of GR genes was higher in *L. c. dorsalis* (84 + 3 pseudogenes) than in *L. c. cuprina* (74 + 6 pseudogenes) with the copy number differences being in a clade related to DmelGr58c and a divergent clade containing nearly two-thirds of the *L. cuprina* GRs, which are most closely related to *DmelGr85a* and *DmelGr47a* (Fig. 7). Both species have two CO_2 receptors, Gr1 and Gr2, that are apparent one-to-one orthologs to the *Drosophila* CO_2 receptors DmelGr21a and DmelGr63a, respectively. Seven sugar receptors (Gr3–Gr9) were identified based on homology to the eight sugar receptors in *D. melanogaster* (Fig. 7). Splice variants were predicted in five GR genes (Gr11, Gr18, Gr24, Gr25, and Gr58) in both genomes, but Gr40 in *L. c. dorsalis* appeared to have alternative splice variants while Gr40 in *L. c. cuprina* appeared to have two full genes suggesting gene duplication (Supplementary File 1).

The ionotropic receptor (IR) family of chemosensory receptors is the most ancient of the genes examined here, evolving from

Fig. 6. Phylogenetic relationships of *L. cuprina* odorant receptors (ORs). Maximum-likelihood tree of *L. c. cuprina* (Lccup), *L. c. dorsalis* (Lcdor), and *D. melanogaster* (Dmel) ORs based on the LG+I+G4 substitution model. The major variation in copy number is in a clade containing four intact copies in *L. c. dorsalis* compared to no intact copies in *L. c. cuprina*. The yellow arrows indicate the 10 highest expressed canonical ORs in the antennae of *L. c. cuprina* based on Wulff et al. (Wulff et al., 2024a). The clades containing one intact ortholog in both *L. cuprina* genomes were collapsed to aid in viewing of the tree. The tree was rooted at the Orco clade.

ionotropic glutamate receptors and being found across the Protostomia (Benton et al., 2009; Croset et al., 2010). Four IRs coreceptors have been identified (Ir8a, Ir25a, Ir76b, and Ir93a) that form a complex with other IRs, referred to as tuning receptors (Ni 2021). The number of ionotropic receptor (IR) genes was slightly greater in *L. c. dorsalis* (78 + 4 pseudogenes) than in *L. c. cuprina* (75 + 3 pseudogenes), with the copy number differences being in the DmeIr75abc clade or in IRs with largely unknown function (*Ir101–Ir148*) (Fig. 8). The highest expressed tuning IRs in *L. c. cuprina* antennae were *Ir31a*, *Ir41a*, *Ir64a*, *Ir84a*, *Ir75a*, *Ir75d-1*, and *Ir76a* (Wulff et al., 2024a).

Odorant binding proteins (OBPs) are a family of soluble proteins classified based on the number of conserved cysteine residues (Graham and Davies, 2002), with those having fewer than six called Minus-C, and those having more than six called Plus-C (Hekmat-Scafe et al., 2002). The OBPs exhibited modest copy number variation among the chemosensory gene families analyzed in the present study. The number of OBP genes in *L. c. cuprina* (54 +3 pseudogenes) was less than *L. c. dorsalis* (57 + 1 pseudogene), with *L. c. dorsalis* having one additional gene, *Obp58* (Fig. 9). Each genome had 11–13 Minus-C (*Obp1*, *Obp15–24*, *Obp50*, *LcdorObp58*), six Plus-C (*Obp51–56*), and two "dimer" OBPs (*Obp25* and *Obp26*) that resemble two OBPs fused into a single transcript. None of these, however, were highly expressed in the *L. c. cuprina* antennae (Wulff et al., 2024a).

4. Discussion and conclusions

Lucilia cuprina consists of two subspecies: *L. c. cuprina* and *L. c. dorsalis,* that exhibit distinctive ecological roles. In Australia, *L. c. dorsalis* is the primary etiological agent of sheep myiasis (flystrike), whereas *L. c. cuprina* does not pose a significant pest threat in Australia, the Americas, or other regions. In this study, we present a chromosome-scale genome assembly for *L. c. cuprina* and an improved assembly for *L. c. dorsalis*, providing a foundation for comparative genomic analyses between these subspecies. This comparative framework offers insights into the genetic underpinnings that likely contribute to their ecological divergence and pest-specific behaviours.

The genome assemblies of *L. c. cuprina* (409.2 Mb) and *L. c. dorsalis* (464.7 Mb) differ in size and contiguity, with *L. c. cuprina* hav-

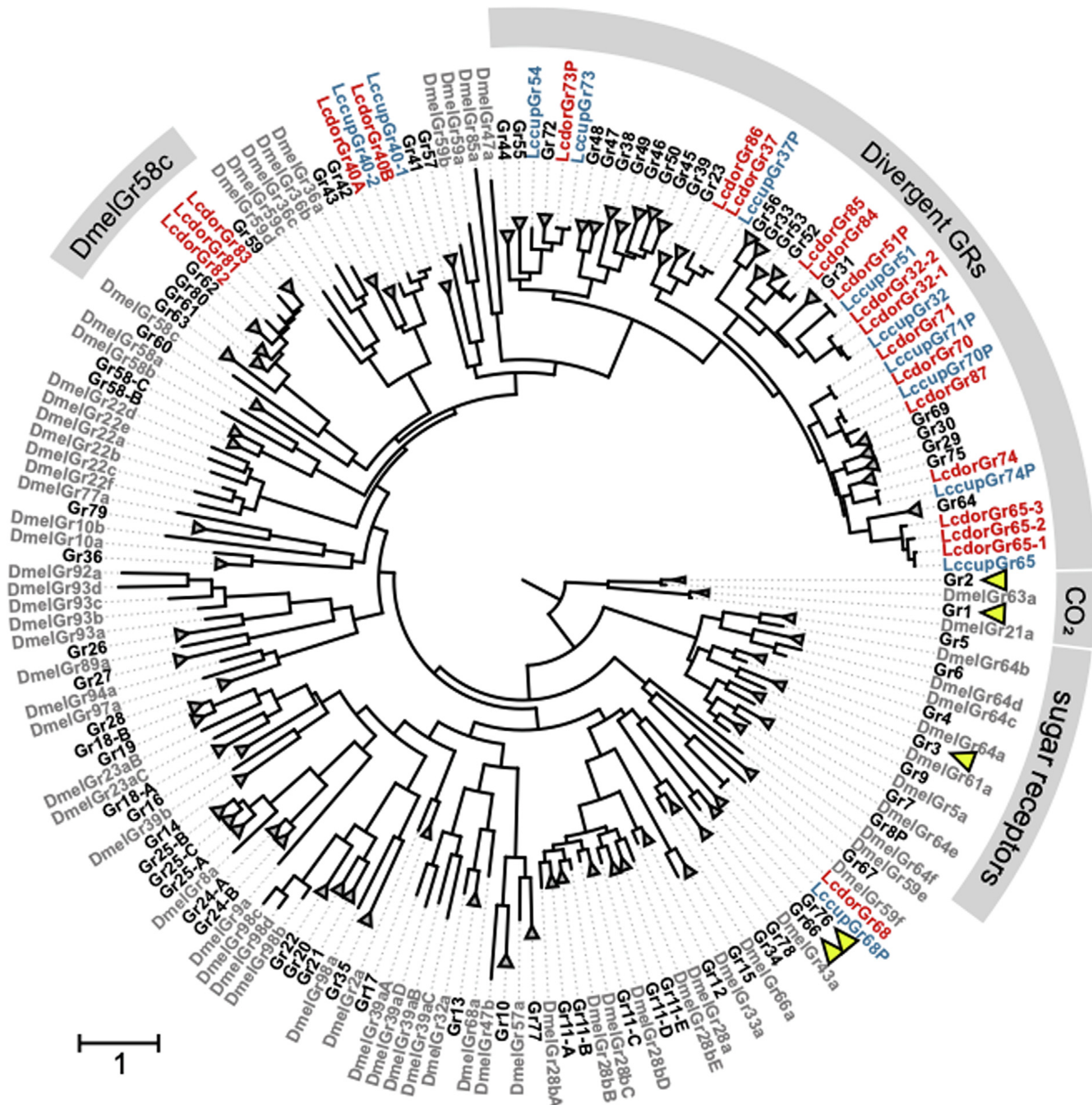

Fig. 7. Phylogenetic relationships of *L. cuprina* gustatory receptors (GRs). Maximum-likelihood tree of *L. c. cuprina* (Lccup), *L. c. dorsalis* (Lcdor), and *D. melanogaster* (Dmel) GRs based on the JTT+F+G4 substitution model. A highly divergent clade with no closely related *D. melanogaster* GRs includes most of the copy number variation, while a clade related to DmelGr58c contained three additional copies in *L. c. dorsalis*. The yellow arrows indicate the five highest expressed GRs in the antennae of *L. c. cuprina* based on Wulff et al. (Wulff et al., 2024a). The clades containing one intact ortholog in both *L. cuprina* genomes were collapsed to aid in viewing of the tree. The tree was rooted at the CO_2 receptors clade.

ing a more contiguous assembly (N50 scaffold length of 71 Mb) compared to *L. c. dorsalis* (N50 of 6.9 Mb). The differences in the number of scaffolds and contigs in *L. c. dorsalis* (560 scaffolds and 32,993 contigs) compared to *L. c. cuprina* (6 chromosomal-length and 8,451 unplaced scaffolds and 27,983 contigs) highlights a disparity in the quality of genome assembly, which is due to differences in sequencing technology and assembly strategies. However, despite this variation in contiguity, the number of protein-coding genes (PCGs) is comparable, with *L. c. dorsalis* having 12,933 PCGs and *L. c. cuprina* having 13,927 PCGs. The differences in gene prediction tools may influence the observed gene content variation or annotations and should be considered when interpreting functional comparisons between the two genomes. However, both species have similar GC content (29.5 %) and high genome completeness, as indicated by BUSCO scores of 97.2 % for *L. c. dorsalis* and 97.1 % for *L. c. cuprina*. The BUSCO scores are similar to those for other fly genomes (Anstead et al., 2015; Sim and Geib, 2017; Olafson et al., 2021; Tandonnet et al., 2023), indicating that both draft genomes are of comparable quality. Although, the assembled genome size for *L. c. cuprina* is significantly smaller than measured by flow cytometry (male 567 Mbp, female 665 Mbp) (Picard et al., 2012), the high BUSCO score suggests that few protein coding genes are missing from the assembly. The assembly is likely missing regions from the largely heterochromatic sex chromosomes. On stained metaphase chromosome spreads the X chromosome is about the size of an autosome (Bedo, 1980) but the X chromosome scaffold (16 Mbp) is much shorter than the autosomal scaffolds (64.5–101.7 Mbp).

The observed genetic variation between the two subspecies indicates significant genomic differentiation and suggests ongoing

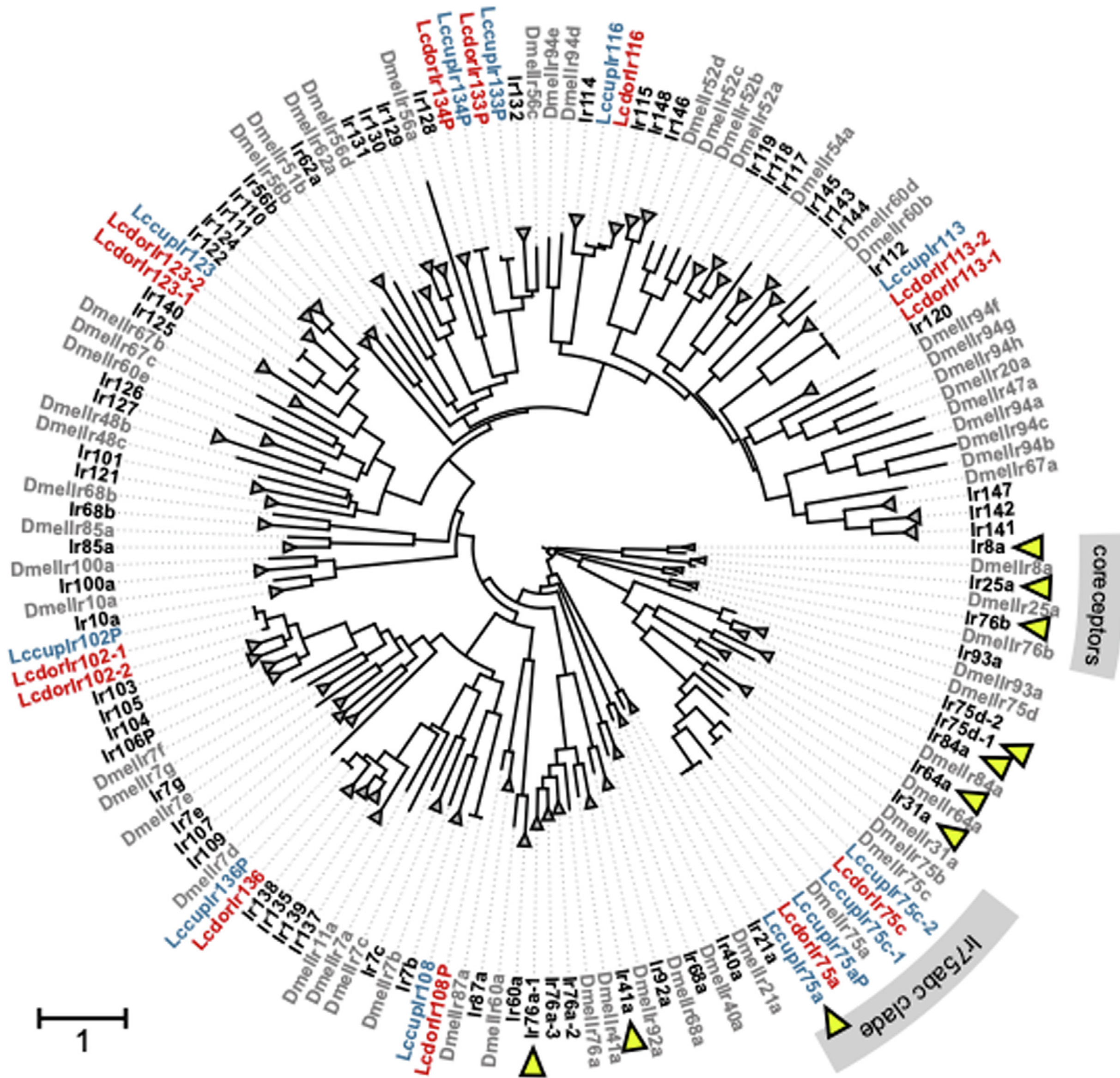

Fig. 8. Phylogenetic relationships of *L. cuprina* ionotropic receptors (IRs). Maximum-likelihood tree of *L. c. cuprina* (Lccup), *L. c. dorsalis* (Lcdor), and *D. melanogaster* (Dmel) IRs based on the LG+F+I+G4 substitution model. *L. c. dorsalis* had additional copies of Ir102, Ir113, and Ir123, while *L. c. cuprina* had one additional copy in the Ir75abc clade. The yellow arrows indicate the 10 highest expressed IRs in the antennae of *L. c. cuprina* based on Wulff et al. (Wulff et al., 2024a). The clades containing one intact ortholog in both *L. cuprina* genomes were collapsed to aid in viewing of the tree. The tree was rooted at the IR co-receptor clades containing Ir8a, Ir25a, Ir76a.

evolutionary divergence. The 29,483 orthogroups observed between these two *Lucilia* subspecies from the OrthoFinder and phylogenetic analyses reflects deep molecular conservation between *L. c. cuprina* and *L. c. dorsalis*. The 10,284 orthogroups shared between these two subspecies represent a core set of conserved genes that likely perform essential biological functions in the genus *Lucilia*. A notable finding was the identification of 543 subspecies-specific orthogroups, containing 1,934 genes, highlighting the evolutionary divergence and subspecies-specific adaptations between *L. c. dorsalis* and *L. c. cuprina*. The fact that *L. c. dorsalis* has a higher proportion of subspecies-specific orthogroups (6.9 %) compared to *L. c. cuprina* (5.1 %) suggests that *L. c. dorsalis* may have undergone more extensive genomic diversification or has retained more lineage-specific genes. These genes could be critical for ecological or behavioural specialisations unique to each subspecies. The presence of novel (as yet uncharacterised) proteins in the two subspecies may indicate that unique, unexplored pathways or evolutionary mechanisms at play. In the subspecies-specific gene set, detoxification-related genes, including CYP450s (*L. c. dorsalis*), glycosyltransferases (*L. c. cuprina*), esterase genes (*L. c. cuprina*), and ATP-binding cassette transporters (*L. c. dorsalis* and *L. c. cuprina*) that contribute to insecticide tolerance in insects were identified. Detoxification genes are essential in metabolising plant secondary compounds and enhancing insecticide tolerance, as observed in *Stenchaetothrips biformis* (Hu et al., 2023). Additionally, subspecies-specific genes in *L. c. dorsalis* included neuronal acetylcholine receptors (AChR) and G-protein coupled receptors (GPCRs), which are known direct targets of several insecticides, including neonicotinoids (Sales et al., 2020; Perry et al., 2021). However, these examples also highlight the need to closely examine each subspecies-specific gene. The AChR genes (KAI8119853.1 and KAI8119854.1) and the mth-like 14 GPCRs (KAI8120093.1 and KAI8120094) are splice variants that encode truncated proteins that may or may not be functional. The absence of a gene in one subspecies compared to the other could be due to poor assembly of the region of the genome that contains the gene in one sub-

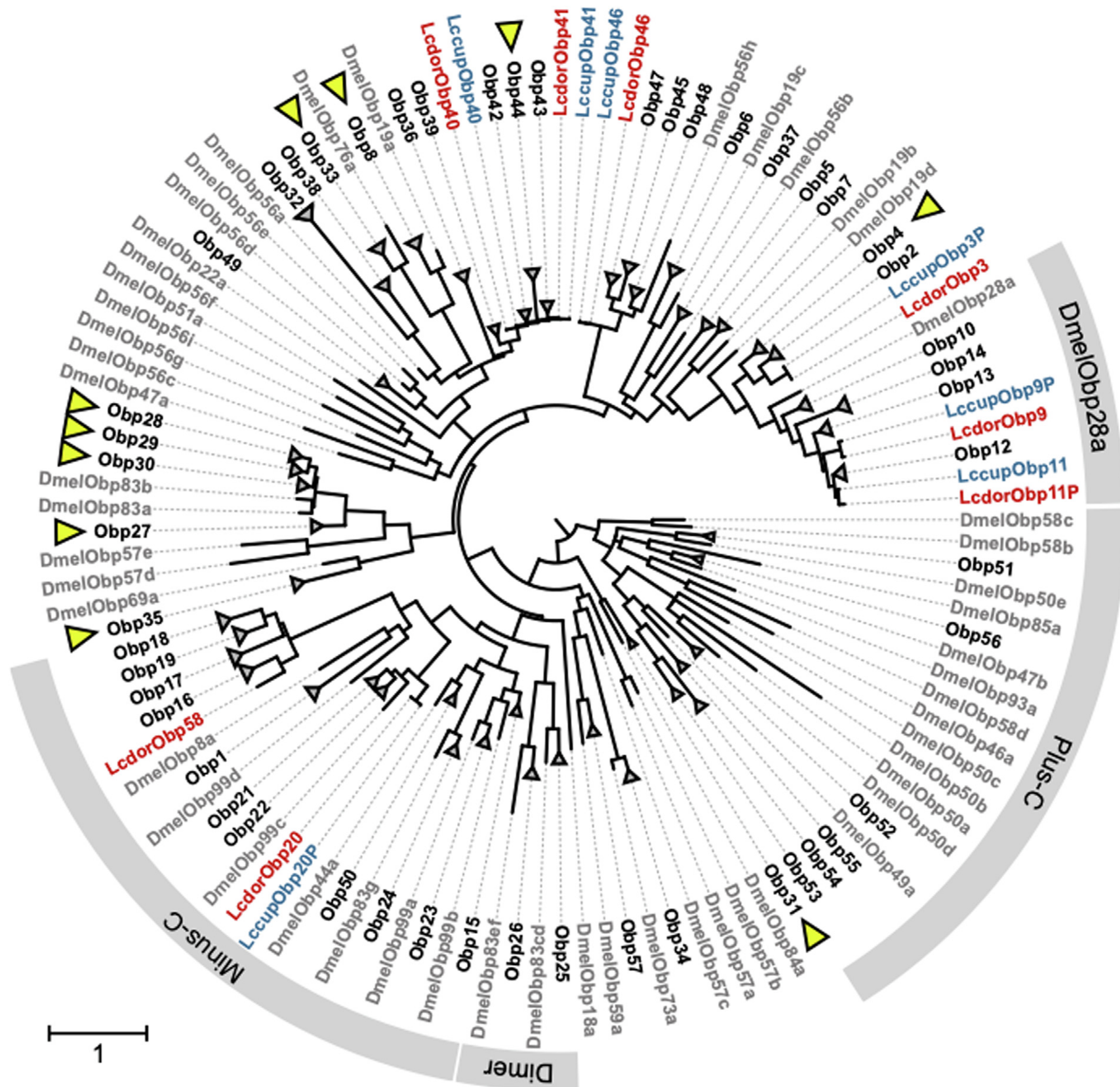

Fig. 9. Phylogenetic relationships of *L. cuprina* odorant binding proteins (OBPs). Maximum-likelihood tree of *L. c. cuprina* (Lccup), *L. c. dorsalis* (Lcdor), and *D. melanogaster* (Dmel) OBPs based on the LG+I+G4 substitution model. The OBPs exhibited moderate copy number variation among the chemosensory genes with *L. c. dorsalis* (56) having two more intact genes than *L. c. cuprina* (54). The yellow arrows indicate the 10 highest expressed OBPs in *L. c. cuprina* antennae based on Wulff et al. (Wulff et al., 2024a). The clades containing one intact ortholog in both *L. cuprina* genomes were collapsed to aid in viewing of the tree. The tree was rooted at the Plus-C clade.

species but not the other. Improved genome assemblies could resolve this issue. Further, as different methods were used for annotating the genomes, the lack of a gene in one subspecies could be because the gene or splice variant was annotated in one subspecies but not the other. In this regard, the Orthofinder analysis identified several GRs specific to *L. c. cuprina*. However, our manual annotation of the chemosensory genes identified orthologs for these GRs in the *L. c. dorsalis* assembly. Consequently, the GRs were removed from the list of *L. c. cuprina*-specific genes. Despite these limitations this analysis has provided promising avenues for future research. It should noted that the observed genomic differences reflect comparisons between two inbred lines that represent two different subspecies, rather than population-level genetic patterns. While these lines are well-characterised laboratory strains originating from distinct geographic and ecological contexts, we acknowledge this limitation that some of the observed variation may represent intraspecific diversity rather than fixed subspecies-level divergence. Future population-wide genomic analyses is essential to validate the extent and nature of these differences.

For many years, organophosphates (OPs) have been used extensively for the management of *L. cuprina,* being applied either as a prophylactic (e.g., sheep dipping and jetting) or as a therapeutic treatment via direct application to infected wounds (Rose et al., 2011). Insecticide-resistant alleles were detected in the a7-esterase enzymes *Rop-1* (diazinon) and *Rmal* (malathion) in the lab strains of *L. c. dorsalis* and *L. c. cuprina,* respectively. These loci should be considered as candidate regions for future validation in larger, population-scale studies to assess their prevalence and functional relevance to insecticide resistance. Diethyl OPs, such as diazinon, have been employed in Australia for the control of *L. cuprina* since 1958, with resistance first reported in 1965

(Shanahan and Hart, 1966). In the absence of alternative insecticide classes until 1979, the prevalence of diazinon resistance escalated to 95 % by the early 1970s and has remained consistently high (Levot, 1995; Rose et al., 2011). The use of diazinon via jetting or dipping was banned in 2007 on operator safety grounds (APVMA, 2006; Sandeman et al., 2014) however, resistance is still present in field populations of *L. c. dorsalis* (McKenzie et al., 1982; Perry et al., 2022). For example, a survey of insecticide resistance levels and their distribution across the sheep producing areas of Australia was recently conducted and revealed that strains of *L. c. dorsalis* from Tasmania and NSW had the minimum resistance ratio (RR) of 8-fold, and the maximum RR of 51-fold, respectively (Sales, 2020). A comparison of these RR ranges with those determined in 1985 (i.e., 5 to 60-fold) provide evidence of the stabilisation of OP resistance (Sales, 2020).

In addition, *L. c. cuprina* has also developed resistance to the dimethyl OP malathion in the USA, even though malathion had primarily been applied to Angora goats for the management of lice, *Bovicola limbrata* rather than blowfly infestations (Taylor et al., 1974; Levot, 2000). However, the frequency of malathion resistance within natural *L. c. cuprina* populations is considerably lower than that observed for diazinon (Smyth et al., 2000). The observed malathion resistance in *L. c. cuprina* populations may be an evolutionary response to extensive aerial malathion applications conducted during the 1980s in California to control outbreaks of the Mediterranean fruit fly, *Ceratitis capitata* (Jackson and Lee, 1985; Dreistadt and Dahlsten, 1986; Mitchell and Saul, 1990). During this campaign, bait sprays containing low concentrations of malathion were repeatedly deployed over nearly 4,000 km^2, including residential areas (Lance et al., 2016). These large-scale spray campaigns likely exerted selective pressure on local insect populations, facilitating the selection of resistant alleles in non-target insect species such as *L. c. cuprina.* Given their non-specific modes of action, insecticides can impact a broad spectrum of non-target organisms, with ecological effects that are often density-independent (Moore, 1967). Diazinon also poses significant risks to wildlife, particularly avian species. During the 1990s, it was responsible for more bird fatalities in the United States than any other pesticide (Cone, 2005). In response to these environmental concerns, the United States implemented a ban on diazinon for residential use in 2005, and the following year, the European Union prohibited all applications of the pesticide (Cone, 2005). These regulatory actions reflect a growing awareness of the unintended ecological consequences associated with pesticide use, including impacts on non-target species and overall ecosystem health.

Members of the family Calliphoridae have been notoriously difficult to resolve phylogenetically and are often regarded a diverse assemblage of lineages in need of major re-classification (Rognes, 1997; Yeates et al., 2007; Kutty et al., 2010; Yan et al., 2021). Further, resolving the taxonomy of the ubiquitous and highly speciose genus *Lucilia* is of particular interest for clear communication in forensic and agricultural settings, and to provide a foundation to better understand how these subspecies differ so significantly in their biology (i.e., necrophagous vs parasitic) despite being so similar genetically. Current efforts to resolve higher-level relationships among blowfly lineages and their relatives have already begun to apply much needed taxonomic changes that better define Calliphoridae (Yan et al., 2021). Here, we compiled data from recently available calliphorid genomic resources to provide a phylogenetic context for further comparative analyses of *L. cuprina* genomic variation.

Our phylogenetic trees of diverse Calliphoridae representatives based on nuclear genomic data (Fig. 4) and of relevant *Lucilia* species/subspecies using a broader taxon sample with anchored hybrid enrichment (AHE) data (Fig. 5), are fully supported and concordant with expected relationships from previous studies (Wells et al., 2007; DeBry et al., 2010; Williams et al., 2016; Yan et al., 2021). The *L. cuprina* subspecies *L. c. cuprina* and *L. c. dorsalis* are placed correctly within the genus and we confirm their position within the broader context of family Calliphoridae. We confirmed *L. sericata* as sister to *L. cuprina*, as found in previous work based on more limited genetic sampling (Wells et al., 2007; DeBry et al., 2010; Williams et al., 2016). The topology recovered by analyses of our genomic dataset is consistent with recent work on the subfamilial and generic relationships within Calliphoridae, specifically in which Luciliinae is sister to Calliphorinae (Yan et al., 2021). Relationships supported by AHE data (Fig. 5) confirm the findings seen in Yan et al. (Yan et al., 2021) on the placement of calliphorid subfamilies, wherein three major lineages diverged: Phumosiinae and Chrysomyinae; Rhinophorinae and Ameniinae sensu (Yan et al., 2021); and Rhiniinae, Bengaliinae, Luciliinae, Calliphorinae sensu Yan et al., 2021, with Luciliinae and Calliphorinae as sister to one another.

The accelerated evolutionary rates observed in both *L. c. cuprina* and *L. c. dorsalis* point to genes potentially involved in adaptations to their respective feeding strategies—necrophagy and parasitism. Notably, among the 245 genes with accelerated evolution in *L. c. cuprina*, we identified one gene, LOC111679291, which encodes a subfamily B ATP-binding cassette (ABC) transporters, which is expressed in adult antennae (Wulff et al, 2024), enriched in L1, L2 larvae (Wulff et al., 2024b) and at other stages. The *Drosophila melanogaster* ortholog is predicted to be localized to the mitochondrial inner membrane and to be involved in oligopeptide export from mitochondrion (Wu et al., 2019; Öztürk-Çolak et al., 2024). In *L. c. dorsalis*, among the 237 genes with accelerated evolution, we highlight the "*Transferrin*" gene, which is involved in iron transport and regulation, suggesting its potential role in blood-feeding (Cardoso-Jaime et al., 2022). Next, we focused on genes exhibiting lower ω values than observed across the phylogeny, hypothesizing that these genes may have acquired new essential roles that are maintained by purifying selection. This functional shift is often observed when genes, after undergoing an initial phase of rapid evolutionary change, become highly constrained due to their involvement in critical biological functions or adaptations that are beneficial to survival and fitness (Hartl and Clark, 1997). In *L. c. cuprina*, we identified "Cytochrome P450", a key enzyme in all insects involved in metabolizing endogenous compounds and xenobiotics, potentially aiding in the detoxification of decomposition-related toxins (Feyereisen, 1999; Scott and Wen, 2001). In *L. c. dorsalis*, more commonly associated with parasitic infestations, we found "Serine proteases (SPs)", a diverse group of enzymes involved in digestion and innate immunity (Li et al., 2005). In *Pyrocoelia rufa* E. Olivier (Coleoptera), for instance, SPs are primarily expressed in the midgut, suggesting a role in protein absorption from the diet (Li et al., 2005).

Our second approach to identify target genes involved performing a GO enrichment analysis for genes with accelerated or slow rates. For accelerated genes in *L. c. cuprina*, most enriched GO terms were associated with the metabolism of biological compounds, potentially involved in degrading toxic substances (Pridie et al., 2020). For the slower-evolving genes, the enriched GO terms were related to ion transport, which could be involved in the excretion of xenobiotics and toxins (Chahine et al., 2012). For *L. c. dorsalis*, GO terms for accelerated genes were largely associated with macromolecule modification and metabolism, processes linked to the regulation of detoxifying enzyme gene expression, a mechanism linked to insecticide resistance (Casida and Durkin, 2013; Sierra et al., 2021). For the slower-evolving genes, enriched GO terms were related to ion organisation and transport, which aligns with the requirements of hematophagous insects, as blood meals

involve substantial metal ion ingestion (Cardoso-Jaime et al., 2022).

In our comparison of the distribution of ω values across all GO terms relative to the whole-genome ω median, we identified "pigmentation" and "extrinsic component of membrane" as the most divergent terms in *L. c. cuprina*, and "carbohydrate derivative binding" and "phenol-containing compound metabolic process" as the most divergent in *L. c. dorsalis*. This approach explores whether different functions and biological processes exhibit distinct evolutionary patterns. In *L. c. cuprina*, most terms were associated with metabolism, amino acid processing, and organic compound transport, processes potentially involved in xenobiotic detoxification (Wu et al., 2019). For *L. c. dorsalis*, the most prevalent GO terms were linked to metabolism, enzymatic activity, and ion transport, consistent with the ingestion of blood and metal by the larvae (Cardoso-Jaime et al., 2022).

The genomes of both *L. cuprina* subspecies contained a similar number of ORs, which is also similar to the 73 intact and five pseudogenes in *C. hominivorax* (Scott et al., 2020). Interestingly, *L. c. dorsalis* had five additional ORs compared to *L. c. cuprina,* which was due to an expansion of the Or37 group (Fig. 6). It will be of interest to determine if any the ORs in this group play an important role in host seeking behaviour of female *L. c. dorsalis*. Of the GRs, the *L. cuprina* subspecies each appear to contain one-to-one orthologs of the *Drosophila* CO2 receptors and seven sugar receptors (Fig. 7). The most striking feature of the GR phylogenetic analysis was the finding that the majority of the GRs were in one clade with no closely related *Drosophila* orthologs. The GRs in this clade were most closely related to the *D. melanogaster Gr85a* and *Gr47a* receptors. While little is known of the function of *DmelGr85a*, *DmelGr47a* is a narrowly tuned receptor for detection of the bitter compound strychnine (Lee et al., 2015). This raises the possibility that the GRs in the divergent clade are receptors that are also narrowly tuned to detect compounds associated with dead or living animals.

Ionotropic receptors (IR) play a large role in olfaction and gustation, especially in amine and acid sensing (Benton et al., 2009; Pitts et al., 2017; Prieto-Godino et al., 2017; Ni, 2021). In addition, they function in thermosensation (Ni et al., 2016) and hygrosensation (Knecht et al., 2017). The *L. cuprina* subspecies had a similar total number of IRs, which was also similar to the total found in *C. hominivorax* (81 + 7 pseudogenes) (Scott et al., 2020). There appears to be an extra copy of the closely related *Ir75a* and *Ir75c* genes in *L. c. cuprina* compared to *L. c. dorsalis* and *D. melanogaster* (Fig. 8). In *D. melanogaster*, *Ir75a* and *Ir75b* are organic acid-sensing receptors expressed in antennae (Prieto-Godino et al., 2017). The *LcupIr75a* gene was one of the most highly expressed IRs in *L. c. cuprina* antennae (Wulff et al., 2024a), suggesting a potential conservation of function.

The odorant binding proteins (OBP) are thought to facilitate the movement of hydrophobic odorants through the sensillum lymph to receptors on the surface of sensory neurons. However, recent studies have found that OBPs are not all necessarily transporters, and the mechanisms in which they function are not well understood (Larter et al., 2016; Pelosi et al., 2018; Ha and Smith, 2022). The number of OBPs found in the *L. cuprina* genomes (54 for *L. c. cuprina* and 57 for *L. c. dorsalis*) is greater than identified in *C. hominivorax,* which has 51 OBPs (Scott et al., 2020). In *D. melanogaster*, *DmelObp28a* was among the most highly expressed in antennae (Larter et al., 2016). However, none of the most closely related OBPs (*Obp9–14*) were expressed over 2 TPM in *L. c. cuprina* antennae (Wulff et al., 2024a). Four of the six OBPs that were expressed at very high levels in antennae (greater than 10,000 TPM), *LcupObp27-30*, are very closely related and form an expanded clade (Wulff et al., 2024a, 2024b) (Fig. 9). The most closely related *Drosophila* genes, *DmelObp83a* and *DmelObp83b* are expressed only in basiconic and trichoid sensilla of the antennae (Larter et al., 2016). Thus, it will be of interest to determine if *LcupObps* 27–30 have specific expression patterns in *L. cuprina* antennae. When considering gain and loss of chemosensory genes between the *L. cuprina* subspecies, it is important to note that the gene models are predictions based on the genome assemblies and consequently any discrepancy could be due to sequencing/assembly errors. Therefore, improved assemblies or targeted genome or transcriptome sequencing is needed to confirm these predictions.

We acknowledge that the subspecific status of *L. c. cuprina* and *L. c. dorsalis*, which are capable of hybridising, complicates the interpretation of observed genomic differences. These differences may reflect a combination of true inter-subspecific divergence and standing intraspecific variation. The potential confounding effects of prolonged laboratory culture and inbreeding must also be considered when interpreting the observed genetic divergence between *L. c. cuprina* and *L. c. dorsalis*. These factors can introduce genetic drift, promote adaptation to controlled conditions, and reduce overall genetic diversity, potentially contributing to divergence that is not solely reflective of natural ecological or evolutionary processes. While our comparative genomic analyses focused on identifying broad genetic differences between ecologically and geographically distinct subspecies, we acknowledge that comprehensive population-level genomic analyses will be crucial in the future to more accurately assess patterns of gene flow and reproductive isolation. Consequently, future studies will incorporate the genomic data from newly collected wild populations, enabling clearer distinction between divergence due to natural environmental variation and laboratory-induced genetic changes. Our analyses of the *L. c. dorsalis* and *L. c. cuprina* genomes have identified genes that may relate to the very different feeding behaviours of the two species (parasitism and necrophagy) and that may play keyroles in sensing their environment. Future functional genetic studies using tools such as CRISPR/Cas9 (Paulo et al., 2019, 2022) could be undertaken to evaluate the importance of the identified genes in fly survival and behaviour. The genomic resources will also greatly facilitate other efforts such as developing engineered strains for genetic suppression of *L. c. dorsalis* (Yan et al., 2020; Novas et al., 2023).

Data Availability

The genome sequence data that support the findings of this study are openly available in GenBank of NCBI at (https://www.ncbi.nlm.nih.gov/) under accession numbers GCF_022045245.1 (Bioprojects PRJNA809378 and PRJNA787202) for *Lucilia cuprina cuprina* and GCA_025217265.1 (Bioproject PRJNA419080) for *Lucilia cuprina dorsalis.*

CRediT authorship contribution statement

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2025.06.001.

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