Article

Morphological and Molecular Characterization Using Genitalia and *CoxI* Barcode Sequence Analysis of Afrotropical Mosquitoes with Arbovirus Vector Potential

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Abstract: Potential arboviral Afrotropical mosquito vectors are underrepresented in public databases of *CoxI* barcode sequences. Furthermore, available *CoxI* sequences for many species are often not associated with voucher specimens to match the corresponding fine morphological characterization of specimens. Hence, this study focused on the characterization of Culicine mosquitoes from South Africa, Mozambique, and Angola and their classification using a complementary approach including a morphological analysis of specimens' genitalia and phylogenetic study based on the analysis of *CoxI* barcode sequences using maximum likelihood and Bayesian phylogenetic inference methods, alongside Median-Joining Network and PCOORD analyses. Overall, 800 mosquitoes (652 males and 148 females) from 67 species, were analyzed. Genitalia from 663 specimens allowed the identification of 55 species of 10 genera. A total of 247 *CoxI* partial gene sequences corresponding to 65 species were obtained, 11 of which (*Aedes capensis*, *Ae. mucidus*, *Culex andersoni*, *Cx. telesilla*, *Cx. inconspicuosus*, *Eretmapodites subsimplicipes*, *Er. quinquevittatus*, *Ficalbia uniformis*, *Mimomyia hispida*, *Uranotaenia alboabdominalis*, and *Ur. mashonaensis*) are, to the best of our knowledge, provided here for the first time. The presence of *Cx. pipiens* ecotypes *molestus* and *pipiens* and their hybrids, as well as *Cx. infula*, is newly reported in the Afrotropical region. The rates of correct sequence identification using BOLD and BLASTn (≥95% identity) were 64% and 53%, respectively. Phylogenetic analysis revealed that, except for subgenus *Eumelanomyia* of *Culex*, there was support for tribes Aedini, Culicini, Ficalbiini, and Mansoniini. A divergence >2% was observed in conspecific sequences, e.g., *Aedeomyia africana*, *Ae. cumminsii*, *Ae. unilineatus*, *Ae. metallicus*, *Ae. furcifer*, *Ae. caballus*, and *Mansonia uniformis*. Conversely, sequences from groups and species complexes, namely, *Ae. simpsoni*, *Ae. mcintoshi*, *Cx. bitaeniorhynchus*, *Cx. simpsoni*, and *Cx. pipiens* were insufficiently separated. A contribution has been made to the barcode library of Afrotropical mosquitoes with associated genitalia morphological identifications.

1. Introduction

Many mosquito species are important vectors of pathogens, including arboviruses, which can cause various febrile, neurological, and hemorrhagic diseases and, therefore, pose a considerable burden on human health and health systems [1]. While currently, the most important arboviruses transmitted by mosquitoes are dengue (DENV), Zika (ZIKV), Chikungunya (CHIKV), and yellow fever (YFV), outbreaks caused by West Nile (WNV), Rift Valley fever (RVFV), and Japanese Encephalitis (JEV) viruses have also been reported in recent years, becoming emerging health problems [2,3].

Mosquitoes (Diptera: Culicidae) are widely distributed throughout the world (except for Antarctica), with 3570 valid species and 130 subspecies thus far documented [1]. The correct identification of mosquito species that may be involved in pathogen transmission is the first step in the surveillance and control of mosquito-borne diseases and has been based on morphological analysis of mainly adult specimens, but also fourth instar larvae [4–8]. Furthermore, several mosquito species can only be identified based on morphological differences in the male genitalia (and occasionally on other male-specific structures), rendering the identification of their female counterparts sometimes unsolved. Nevertheless, the characteristics of male genitalia are structural, allowing accurate and reliable species identification, in addition to being less susceptible to general body damage that is so common in field samples. However, genitalia dissection is a fine and tedious process that requires specific and specialized training [9]. Furthermore, some mosquitoes form closely related, morphologically indistinguishable, cryptic species complexes, with each species having ecological and host preferences and reproductive isolation, constituting biological individual taxa. To overcome the difficulty in their identification, nucleic acids-based molecular identification methods are used for, for example, members of multiple *Anopheles* species complexes [10] and *Culex (Culex) pipiens* complex members [11].

So far, despite the medical importance of diseases such as dengue, yellow fever, West Nile fever, Zika, and Rift Valley fever, studies aimed at the molecular identification of vectors of arboviruses of African origin [12–16] are limited compared to those regarding the analysis of malaria vectors, or even arbovirus vectors of non-African origin [17–28]. The molecular identification of many species occurring in countries such as South Africa, Mozambique, and Angola that have high mosquito and viral richness are not available [12,29–32]. Cytochrome c oxidase subunit I (*CoxI*) barcode sequences of many Afrotropical mosquito vectors of arboviruses are lacking due to the underrepresentation of specimens in the largest public genomic sequence databases most frequently searched (BOLD and GenBank). Examination of the global representation of *CoxI* barcode Culicidae species sequences in BOLD clearly reveals the underrepresentation of African-derived taxa (https://www.boldsystems.org/index.php/Public_SearchTerms, accessed on 12 May 2022). Furthermore, it is essential to have reliable and comprehensively annotated reference databases of verified sequences that can be used for comparison for species identification [20]. Phylogenetic analyses based on some GenBank/BOLD records have suggested that some partial genomic sequences obtained from mosquitoes have been incorrectly assigned, a type of error that has already been identified in studies based on the *CoxI* marker [25] and internal transcribed spacer of nuclear ribosomal DNA (*ITS*) [33].

The objectives of this work were: (i) to morphologically characterize Afrotropical mosquitoes of the Culicinae subfamily, focusing on the analysis of genitalia of adult specimens, in order to have morphological vouchers associated with a matching mitochondrial *CoxI* sequence to be obtained sequently; (ii) to perform a phylogenetic reconstruction that would allow the identification of the sequences obtained in this work, but that would

also (iii) shed light on the agreement between phylogenetic tree topology and the current morphology-based taxonomic arrangement.

2. Materials and Methods

2.1. Mosquito Sampling and Preparation of Male Mosquito Genitalia

The mosquito collection analyzed in this work represented a convenience sample comprising specimens previously collected in three countries in southern Africa (Mozambique, South Africa, and Angola; Supplementary Material-SIV (File S-IV), Figure S1) between 2014 and 2018, within the scope of various scientific projects related to arbovirus detection and epidemiology assessments of arboviruses. After collection and subsequent transportation to the Institute of Hygiene and Tropical Medicine | NOVA University Lisbon (IHMT | NOVA), these mosquitoes, listed in Supplementary Material-I (File S-I), Table S1, were kept dehydrated in silica gel tubes at room temperature.

All mosquitoes were classified according to species, species complex, or species group (where possible) based on the analysis of their morphological features, following the keys of Edwards [4], Jupp [5], and Harbach [34]. The classification of the Aedini tribe followed that of Wilkerson et al. [35] and taxa nomenclature as in https://mosquito-taxonomic-inventory.myspecies.info/valid-species-list# (accessed on 12 May 2022).

The genitalia of all male, and some female, specimens were dissected and slide-mounted for careful examination. The terminal part of the mosquito abdomen was sectioned at the level of segment VII/VIII and immersed in Marc André's solution for a minimum of 7 days at room temperature. Afterward, mosquito genitalia were placed on a slide with a drop of a polyvinyl-chloral-formo-phenol medium, dissected under a stereomicroscope, and covered with a coverslip [14]. Analysis of the different structures of the mosquito genitalia and (sometimes) of maxillary palps, were carried out using an Olympus microscope (BX5,1) and their identification and naming of parts followed the nomenclature of Harbach and Knight [9]. Photographs were taken with an Olympus SC30 digital camera and processed with the Zerene Stacker program (https://www.zerenesystems.com/, accessed on 12 May 2022). In *Culex* subgenus *Oculeomyia*, we relied on the description by Sirivanakarn [36] and Harbach [34] to confirm the identification based on the genitalia.

2.2. DNA Extraction, Partial Amplification of CoxI, and Culex Pipiens Complex Molecular Identification

Total genomic DNA was extracted from mosquito legs and abdomens, as previously described [14]. The barcode N-terminal region of the *CoxI* gene was amplified using the specific primers (LCO1490 and HCO2198), using reaction conditions described by Folmer et al. [37]. The amplified products of 658 bp were visualized under UV illumination after electrophoresis in 2% agarose gels. Whenever a specific amplification product was not observed, to obtain a *CoxI*-specific amplicon, an alternative strategy was used. This entailed the use of primers LCO1490 and TL2-N-3014 and the thermal profile previously described by Tchouassi et al. [38]. In case unsuccessful amplifications prevailed, a final attempt called for the design of new primers using multiple alignments of *CoxI* nucleotide sequences downloaded from the GenBank genomic database. These sequences were aligned using MAFFT v7 (https://mafft.cbrc.jp/alignment/server/, accessed 10 November 2021), and these alignments served as a starting point for the design of degenerate primers using the primer design-M tool (https://bio.tools/primerdesign-m, accessed 10 November 2021). The chosen primers (C_degF 5'-ACWTTATAYTTYATTTTYGG-3' and C_degR 5'-GTTARWARTAT-WGTAATWGC-3') were used at a final concentration of 500 nM in 20 μL PCR reactions containing 10 μL NZYTaq II 2x Green Master Mix (NYTech, Portugal), 2 μL of a 1:10 dilution of the original DNA extract, and 6 μL of nuclease-free water. The amplification conditions included one denaturation step at 95 °C for 5 min, followed by 40 cycles of amplification (denaturation: 30 s at 95 °C; annealing: 40 s at 43 °C; extension: 1 min at 72 °C) and a final extension step for 5 min at 72 °C.

A multiplex PCR assay that targets species-specific polymorphisms at the intron-2 of the acetylcholinesterase gene intron-2 (*Ace-2*) sequence of *Cx. pipiens* Linnaeus, 1758 and *Cx. quinquefasciatus* Say, 1823 was carried out with primers B1246s, ACEpip, and ACEquin, as described by Smith and Fonseca [11], yielding a PCR product of 610 bp for *Cx. pipiens* and 274 bp for *Cx. quinquefasciatus*. Differentiation of the *Cx. pipiens* ecotype *molestus* and *Cx. pipiens* ecotype *pipiens* followed the analysis of the CQ11 microsatellite flanking region, described by Bahnck and Fonseca [39], yielding a PCR-product approximately 200 bp in size for *Cx. pipiens* form *pipiens* and 250 bp for form *molestus*.

2.3. Amplicon Sequencing and Nucleotide Sequence Analyses

The amplified PCR products corresponding to partial sequences of the *CoxI* gene from each of the analyzed samples were purified and sequenced by the Sanger method (STABVida, Lda. 2825-182 Caparica, Portugal) using primers LCO1490 or C_deg_F, and the respective reverse primers when the obtained chromatogram lacked in quality. The sequences obtained were edited using the Chromas tool version 2.6.6 (https://technelysium.com.au/wp/, accessed on 10 November 2021). Low-quality sequences were excluded during the editing process. In these cases, a new amplification was performed from the same DNA extract. The purification and sequencing of the obtained amplification products were also repeated, as described above. All amplification products were sequenced, which ranged from 399–661 nucleotides. However, for phylogenetic and divergence analysis, only sequences greater than 500 nucleotides were considered.

The search for homologous sequences available in publicly accessible genomic databases (GenBank/ENA/DDBJ) was performed both with the BLASTn tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 10 November 2021) and the taxonomy search engine in the BOLDSystems v4 database (https://www.boldsystems.org/index.php/IDS_OpenIdEngine, accessed on November 2021). These same tools were used for the identification/confirmation of the identity of our sequences. Multiple sequence alignments were constructed using the G-INS-i iterative refinement method as implemented in MAFFT v7. The obtained alignments were treated with Gblocks (http://phylogeny.lirmm.fr/phylo_cgi/one_task.cgi?task_type=gblocks, accessed 10 November 2021) after selecting the most permissive editing options. The evaluation of the phylogenetic signal of all used sequence datasets was carried out using the likelihood-mapping method, as implemented in the TREE-PUZZLE software [40].

For the phylogenetic sequence analysis, two different approaches were explored: the Maximum Likelihood optimization criterion (ML) and a Bayesian phylogenetic inference-based approach. For both, the first step of the analysis involved the choice of the best nucleotide substitution model to be used (GTR + Γ, GTR + I or GTR + Γ + I models: GTR-General Time Reversal; Γ-Gamma distribution; proportion I of invariant sites), using the IQtree software [41], which was also used for ML phylogenetic reconstruction. The topological support of the branches in the obtained trees was assessed with bootstrap analysis and an approximate likelihood ratio test [aLRT], also implemented in Iqtree. In either case, 1000 replicates of the original sequence data were used, and bootstrap or aLRT values $\geq$ 75 (% of the total number of replicates) were considered as indicating strong topological support.

Bayesian phylogenetic analyses were carried out using BEAST v1.10.4 software [42], using the same sequence data sets and evolutionary models adopted for the ML analyses. The Bayesian analyses consisted of two independent Markov chain Monte Carlo (MCMC) runs until 1×10^8 states had been sampled at every 10,000th MCMC step (10% of which were later discarded as burn-in). In all cases, chain convergence was assessed using Tracer software v1.7.1 (http://beast.bio.ed.ac.uk/tracer, accessed on 10 November 2021), which was also used to check for an adequate effective sample size (ESS) higher than 200 (after the removal of the burn-in). The tree distribution was summarized using TreeAnnotator software v1.8.3 as a maximum clade credibility (MCC) tree, using median heights as the node heights in the tree. All the phylogenetic trees were visualized using FigTree v1.4.2

software (http://tree.bio.ed.ac.uk/software/figtree/, accessed 10 November 2021). At specific branches, a posterior probability value of ≥ 0.80 was considered as indicating strong topological support. In all trees, the sequence of the species *An. neomaculipalpus* Curry, 1931 (KM592986.1) was used as the outgroup. The trees obtained with maximum credibility (product of the Bayesian analysis) were selected to depict a topological organization of the branches more compatible with *a priori* taxonomic expectations. Specific branches were labeled with one to three "*" signs, according to the number of phylogenetic construction methods/tests that confirmed such topology (aLRT and bootstrap/ML + posterior probability/Bayesian analyses). The original trees can be found in Supplementary Material-III (File S-III).

The average intraspecific and interspecific genetic variation were calculated using genetic distances corrected with the Kimura 2-parameter model (K2P), as implemented in the MEGA X software.

Median Joining networks analysis was performed using SplitsTree5 5.0.0_alpha application with default options [43] for computing unrooted phylogenetic networks from alignments of sequences. The Neighbor Net method [44] was used (default options) to obtain compatible splits, and the Splits Network Algorithm method [45] was used (default options) to obtain split networks. Principal coordinates analysis was also carried out using the software available on the platform (https://www.hiv.lanl.gov/content/sequence/PCOORD/PCOORD.html, accessed on 10 April 2022).

3. Results

3.1. Mosquito Identification: Morphological and Molecular

A total of 800 mosquitoes, comprising 652 (81.5%) males and 148 (18.5%) females, were analyzed in this study. These included 73 specimens from Angola, 515 from South Africa, and 212 from Mozambique, representing 67 species belonging to 10 genera: *Aedeomyia* (2), *Aedes* (28), *Coquillettidia* (3), *Culex* (24), *Eretmapodites* (2), *Ficalbia* (1), *Lutzia* (1), *Mansonia* (2), *Mimomyia* (2), and *Uranotaenia* (2) (File S-I, Table S1).

Of these, genitalia from 652 male and 11 female specimens were dissected and their analysis confirmed the identification of 55 species (File S-IV, Table S1), a photographic record of which can be found in Supplementary Material-II (File S-II). The respective slides are deposited in the IHMT|NOVA Insect Collection.

From a subsample of genitalia-confirmed male *Cx. pipiens* and *Cx. quinquefasciatus* and females from the *Cx. pipiens* complex, *Ace2* multiplex PCR allowed us to confirm 8 specimens (2 females and 6 males) as *Cx. pipiens* and 18 as *Cx. quinquefasciatus* (7 females and 11 males; File S-IV, Figure S2a). No hybrids were identified and four samples failed to react. Multiplex PCR for the CQ11 microsatellite flanking region identified one of the *Cx. pipiens* as the *pipiens* ecotype (EM305), two as the *molestus* ecotype (EM326 and EM332), and four as hybrids of the two ecotypes (EM300, EM302, EM303, EM304), while no amplification product was obtained for one male *Cx. pipiens* (EM306) (File S-IV, Figure S2b).

The amplification of the *CoxI* gene was successful in 247/333 specimens (74.2%). The majority (*n* = 184) of the *CoxI* amplicons were obtained with the Folmer et al. [37] protocol, while the remaining 63 sequences were obtained either with the Tchouassi et al. protocol (*n* = 14) [38] or using the degenerate primers/protocol here described (*n* = 49). A total of 65 species were identified through molecular analysis (File S-IV, Table S2). Not all species could be identified by both methods as in some, no males were available, and in others, no amplification was obtained, leaving the total number of species identified by either method as 67. Only 64% of the sequences obtained were correctly identified by the BOLD tool, i.e., corresponding to the genitalia-confirmed species, and 53% shared $\geq 95\%$ identity with a given species-specific sequence using the BLASTn tool. For eleven of these species, and as far as we could ascertain, partial *CoxI* sequences are provided here for the first time. These species include *Ae. (Albuginosus) capensis* Edwards, 1924; *Ae. (Mucidus) mucidus* (Karsch, 1887); *Cx. (Culex) andersoni* Edwards, 1914; *Cx. (Cux.) telesilla* de Meillon and Lavoipierre, 1945; *Cx. (Eumelanomyia) inconspicuosus* (Theobald, 1908); *Er. subsimplicipes*

Edwards, 1914; *Er. quinquevittatus* Theobald, 1901; *Fi. uniformis* (Theobald, 1904); *Mi. (Mim) hispida* (Theobald, 1910); *Ur. (Uranotaenia) alboabdominalis* Theobald, 1910; and *Ur. (Pseudoficalbia) mashonaensis* Theobald, 1901 (the male genitalia of which are presented in Figure 1).

Figure 1. Genitalia of male mosquito species whose partial sequence of the *CoxI* gene was obtained for the first time in this study: (**a**,**b**) *Ae.* (*Alb.*) *capensis*, (**c**,**d**) *Ae.* (*Muc.*) *mucidus*, (**e**) *Er. subsimplicipes*, (**f**) *Er. quinquevittatus*, (**g**,**h**) *Cx.* (*Cux.*) *andersoni*, (**i**,**j**) *Cx.* (*Cux.*) *telesilla*, (**k**,**l**) *Cx.* (*Eum.*) *inconspicuosus*, (**m**) *Fi. uniformis*, (**n**) *Mi. hispida*, (**o**) *Ur.* (*Ura.*) *alboabdominalis*, (**p**) *Ur.* (*Pfc.*) *mashonaensis*. Most photographs represent the whole genitalia, with the exception of (**b**) detail of gonostylus, (**d**) detail of basal dorsomesal lobe, claspettes, and proteger, (**g**,**i**,**k**) phallosome, and (**h**,**j**,**l**) gonocoxite with gonostylus.

3.2. Mosquito Identification Using Phylogenetic Reconstruction

3.2.1. Genus *Aedeomyia*

Aedeomyia sequences were grouped phylogenetically according to their subgenera (Figure 2). *Aedeomyia* (*Aedeomyia*) *africana* Neveu-Lemaire, 1906 from Mozambique (File S-II Figure S1) was grouped according to a conspecific sequence from Malawi and another from Madagascar, *Ad.* (*Ady*) *madagascarica* Brunhes, Boussès & da Cunha Ramos, 2011. Those from Kenya formed their own clade with a divergence of 6.9% ± 1.3 between the two *Ad. africana* clades (Figure 2 and Supplementary Material File File S-IV, Table S3). *Aedeomyia* (*Lepiothauma*) *furfurea* (Enderlein, 1923), both from Mozambique and South Africa, formed a strong clade. The divergence between these two species was >10%. Networks and PCOORD analyses agreed with that topology (File S-IV, Figure S3).
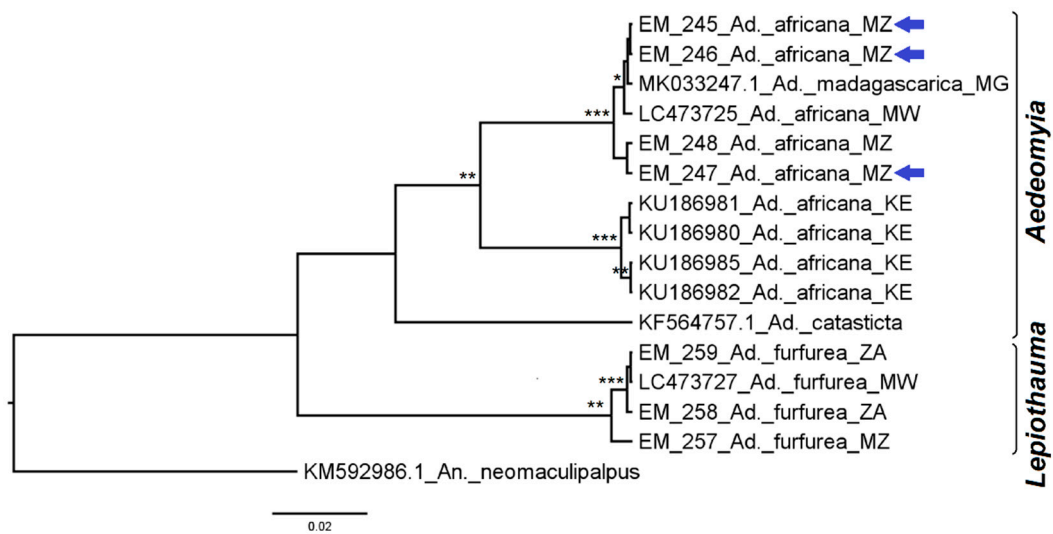
Figure 2. Phylogenetic analysis of 15 partial *coxI* nucleotide sequences from *Aedeomyia* mosquitoes. At specific branches, the number of * indicates the tree topological support revealed by the different phylogenetic reconstruction methods, assuming relevant bootstrap and aLRT values above 75% and posterior probability values above 0.80. The sequences obtained in this work are designated with the "EM" code, while those with associated genitalia are indicated with horizontal blue arrows. Reference sequences downloaded from the public databases are shown by their respective access codes (Boldsystems) or accession numbers (GenBank), as well as the country of origin [South Africa (ZA), Madagascar (MG), Malawi (MW), Mozambique (MZ), Kenya (KE)]. Vertical lines mark the *Aedeomyia* and *Lepiothauma* subgenera.

3.2.2. Genus *Aedes*

Aedes sequences formed two main clusters, with species within subgenera *Mucidus* and *Ochlerotatus* forming a cluster separated from species representing all the other *Aedes* subgenera (Figure 3).
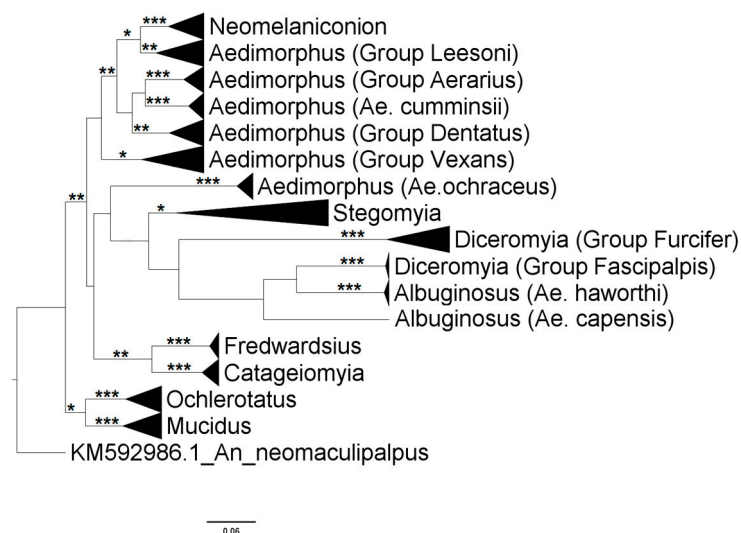

Figure 3. Phylogenetic analysis of 172 partial *coxI* nucleotide sequences from *Aedes* mosquitoes. At specific branches, the number of * indicates the tree topological support revealed by the different phylogenetic reconstruction methods, assuming relevant bootstrap and aLRT values above 75% and posterior probability values above 0.80. In the collapsed branches are the species of the subgenera and/or informal groups of the subgenera.

Species in the subgenus *Neomelaniconion* formed a single, monophyletic, strongly supported clade (Figure 4) in which *Ae. (Neo.) mcintoshi* Huang, 1985 (File S-II Figure S16), *Ae. (Neo.) unidentatus* McIntosh, 1971, and *Ae. (Neo.) circumluteolus* (Theobald, 1908) (File S-II Figure S15) were grouped in a clade with a variation of 1.2% ± 0.3 that overlapped the interspecific divergence (1.1–1.4%) (File S-IV, Table S4). *Aedes (Neo.) lineatopennis* (Ludlow, 1905) formed a sister clade, showing a divergence with the other species ≥ 5.4%. Networks and PCOORD analyses supported these results (File S-IV, Figure S4).
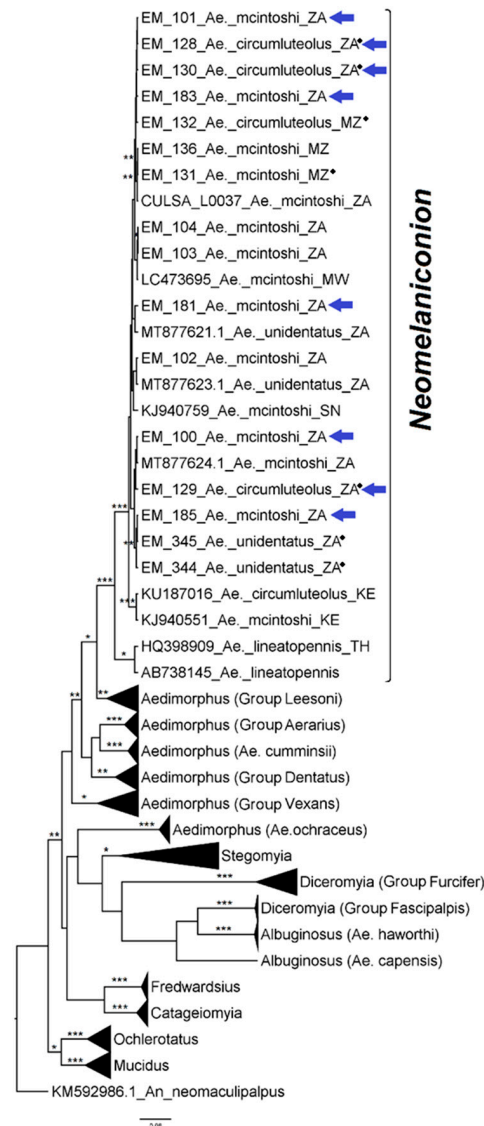

Figure 4. Phylogenetic analysis of 172 partial *coxI* nucleotide sequences from mosquitoes of the genus *Aedes*, presenting the subgenus *Neomelaniconion*. At specific branches, the number of * indicates the tree topological support revealed by the different phylogenetic reconstruction methods, assuming relevant bootstrap and aLRT values above 75% and posterior probability values above 0.80. The sequences obtained in this work are indicated with the "EM" code, and those with associated genitalia are indicated by horizontal blue arrows. The sequences downloaded from GenBank and Boldsystems are indicated by their respective accession numbers and access codes (respectively); the symbol "." after the code of our sequence indicates that said sequence was not identified by Boldsystems. Their country of origin [South Africa (ZA), Malawi (MW), Mozambique (MZ), Kenya (KE), Senegal (SN), Thailand (TH)] are also indicated. The vertical line marks the subgenus *Neomelaniconion* and the collapsed branches indicate the species of the subgenera and/or informal groups of the subgenera.

Most sequences of taxa in the subgenus *Aedimorphus* formed a polyphyletic clade, separating into subclades according to their morphologically based groupings designated by McIntosh [46] (Figure 5). However, *Ae. (Adm.) cumminsii* (Theobald, 1903) sequences from Kenya, Guinea, and Senegal shared an inter-group variation that ranged from 0.7–2.3%, according to the origin (File S-IV, Table S5), forming a clade distant from the conspecific sequences from South Africa, which joined the Dentatus group, *Ae. (Adm.) dentatus* (Theobald, 1904) (File S-II Figure S3) and *Ae. (Adm.) pachyurus* Edwards, 1936, to which they belong. The divergence between these two groups of *Ae. cumminsii* was ≥7.4%. Networks and PCOORD analyses corroborated this finding (File S-IV, Figure S5).
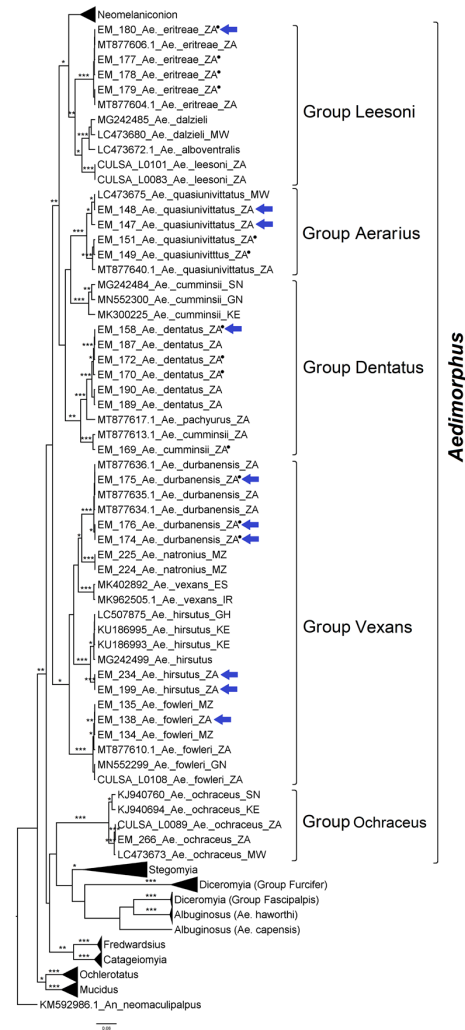

Figure 5. Phylogenetic analysis of 172 partial *coxI* nucleotide sequences from mosquitoes of the genus *Aedes*, presenting the subgenus *Aedimorphus*. At specific branches, the number of * indicates the tree topology support revealed by the different phylogenetic reconstruction methods, assuming relevant bootstrap and aLRT values above 75% and posterior probability values above 0.80. The sequences obtained in this work have the "EM" code and those with associated genitalia are indicated by horizontal blue arrows. The sequences downloaded from GenBank and Boldsystems are indicated by their respective accession numbers and access codes (respectively); the symbol "." after the code of our sequence indicates that said sequence was not identified by Boldsystems. Their country of origin [South Africa (ZA), Spain (ES), Ghana (GH), Guinea (GN), Iran (IR), Malawi (MW), Mozambique (MZ), Kenya (KE), Senegal (SN)] are also indicated. The vertical lines mark the informal groups and the subgenus *Aedimorphus*; the collapsed branches are the species of the subgenera and/or informal groups of the subgenera.

The *Stegomyia* subgenus formed a monophyletic clade in which most species formed well-supported species group clades (Figure 6). One exception was within the Simpsoni group where *Ae. (Stg.) simpsoni* (Theobald, 1905) and *Ae. (Stg.) bromeliae* (Theobald, 1911) formed a single clade with a variation of 1.1% ± 0.3, while the interspecific divergence of the species in the clade was 1.3% ± 0.4. *Ae. (Stg.) unilineatus* (Theobald, 1906) formed two monophyletic sister clades comprising sequences from either South Africa (File S-II Figure S20) or Pakistan, with a global intraspecific variation of 3.4% ± 0.6 and an inter-clade variation of 5.3% ± 0.9. Similarly, sequences from *Ae. (Stg.) metallicus* (Edwards, 1912) (Figure S-II S19) formed two monophyletic sister clades, with an inter-clade distance of 7.4% ± 1.0. These results were corroborated by the networks and PCOORD analyses, evidencing the near lack of separation of *simpsoni/bromeliae*, wider separation for the two groups of *unilineatus*, and even greater separation for *metallicus* (File S-IV, Figure S6).
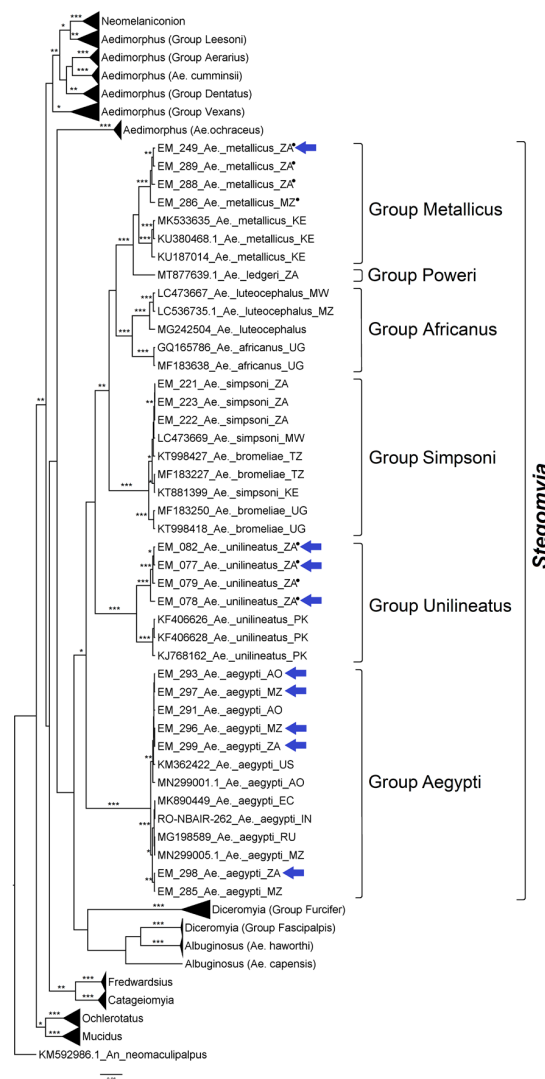

Figure 6. Phylogenetic analysis of 172 partial *coxI* nucleotide sequences from mosquitoes of the genus *Aedes*, presenting the subgenus *Stegomyia*. At specific branches, the number of * indicates the tree topological support revealed by the different phylogenetic reconstruction methods, assuming relevant bootstrap and aLRT values above 75% and posterior probability values above 0.80. The sequences obtained in this work are indicated with the "EM" code and those with associated genitalia are indicated by horizontal blue arrows. The sequences downloaded from GenBank and Boldsystems are indicated by their respective accession numbers and access codes (respectively); the symbol "." after the code of our sequence indicates that said sequence was not identified by Boldsystems. Their country of origin [South Africa (ZA), Angola (AO), United States (US), Ecuador (EC), Russian

Federation (RU), India (IN), Malawi (MW), Mozambique (MZ), Pakistan (PK), Kenya (KE), Tanzania (TZ), Uganda (UG)] are also indicated. The vertical lines mark the informal groups and the subgenus Stegomyia; the collapsed branches are the species of the subgenera and/or informal groups of the subgenera.

Subgenus *Diceromyia* was paraphyletic, but the two species included—*Ae. (Dic.) furcifer* (Edwards, 1913) and *Ae. (Dic.) fascipalpis* (Edwards, 1912), both represented by specimens from South Africa (File S-II Figures S11 and S12)—formed species-specific clades with strong support (Figure 7), confirmed in networks and PCOORD analyses (File S-IV, Figure S7).
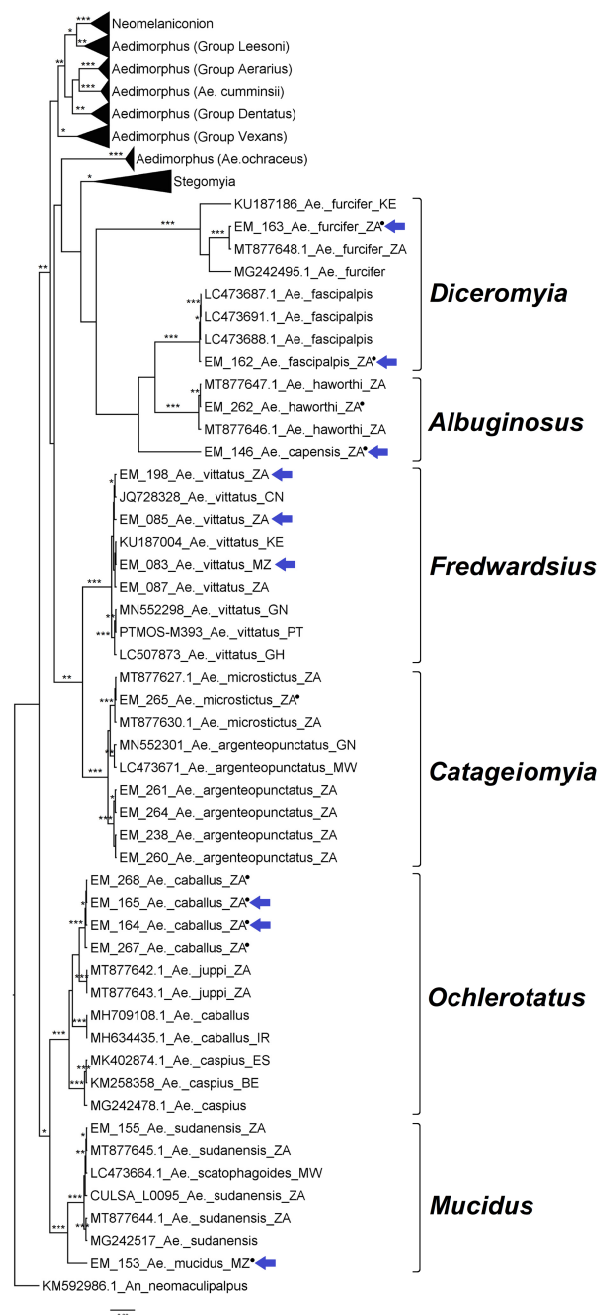

Figure 7. Phylogenetic analysis of 172 partial *coxI* nucleotide sequences from mosquitoes of the genus *Aedes*, presenting the subgenera *Diceromyia*, *Albuginosus*, *Fredwardsius*, *Catageiomyia*, *Ochlerotatus*, and

Mucidus. At specific branches, the number of * indicates the tree topological support revealed by the different phylogenetic reconstruction methods, assuming relevant bootstrap and aLRT values above 75% and posterior probability values above 0.80. The sequences obtained in this work are indicated with the "EM" code and those with associated genitalia are indicated by horizontal blue arrows. The sequences downloaded from GenBank and Boldsystems are indicated by their respective accession numbers and access codes (respectively); the symbol "." after the code of our sequence indicates that said sequence was not identified by Boldsystems. Their country of origin [South Africa (ZA), Belgium (BE), China (CN), Spain (ES), Ghana (GH), Guinea (GN), India (IN), Iran (IR), Malawi (MW), Mozambique (MZ), Portugal (PT), Kenya (KE)] are also indicated. The vertical lines mark the subgenera shown; the collapsed branches are the species of the subgenera and/or informal groups of the subgenera.

Ochlerotatus sequences formed a strong clade, with equally strong paraphyletic sub-clades; in these, *Ae. (Och.) caballus* (Theobald, 1912) (File S-II Figure S17) and *Ae. (Och.) juppi* McIntosh, 1973, both from South Africa, segregated into closer subclades, while *Ae. caballus* from Iran formed a separate cluster (Figure 7). Intraspecific variance within each of the three groups was low ($\leq 0.5\% \pm 0.2$); interspecific divergence between *Ae. caballus* and *Ae. juppi* from SA was $2.8\% \pm 0.7$, and *Ae. caballus* from Iran had a divergence $\geq 3.6\% \pm 0.8$ to either *Ae. caballus* or *Ae. juppi* from SA (File S-IV, Table S6). Networks and PCOORD analyses also placed *Ae. caballus* and *Ae. juppi* from SA closer to one another and farther apart from *Ae. caballus* from Iran (File S-IV, Figure S8).

The clade defining the subgenus *Mucidus* was strongly supported. *Aedes (Muc.) sudanensis* (Theobald, 1908) and *Ae. (Muc.) scatophagoides* (Theobald, 1901) were grouped in a single monophyletic cluster with an intra-clade variation of $0.9\% \pm 0.3$ and an interspecific divergence of $0.6\% \pm 0.2$. The *Ae. mucidus* sequence from a Mozambique specimen segregated away from all *Ae. scatophagoides* with a divergence of $7.5\% \pm 1.2$. Similarly, networks and PCOORD analyses placed *Ae. mucidus* sequences far from the *sudanensis* and *scatophagoides*, which were either pooled in an unsolved group or distributed along a single "dimension" without segregation (File S-IV, Figure S9).

3.2.3. Genus *Eretmapodites*

Eretmapodites sequences formed monophyletic clades separating the various species analyzed. Based on morphological features of male genitalia, *Er. intermedius*, *Er. subsimilicipes* (File S-II Figure S22), and *Er. chrysogaster* were very similar and considered members of the "Chysogaster group," and separated quite distinctly from a clade consisting of a sequence of *Er. quinquevittatus* from Mozambique (File S-II Figure S21), which had quite different male genitalia and adult scutal patterns and a GenBank sequence denoted as *Er. silvestris* Ingram and de Meillon, 1927 (Figure 8). *Eretmapodites subsimplicipes* showed no intraspecific variation and diverged from *Er. quinquevittatus* by $9.3\% \pm 1.5$ (File S-IV, Table S7). Similar results were obtained with networks and PCOORD analyses (File S-IV, Figure S10).

3.2.4. Genera *Culex* and *Lutzia*

Sequences from the genus *Culex* segregated into a highly polyphyletic topology, where most species of subgenus *Culex* segregated into clusters intermingled with members of other subgenera. Two major clades with support of one of the three methods were formed; the first contained species of the subgenus *Culex*, namely, some members of the groups Pipiens, Sitiens, the subgroup Vishnui, and the subgenus *Oculeomyia*; the second clade contained species of the subgenus *Culex*, namely, members of the Pipiens and Duttoni groups and the subgenus *Culiciomyia*. Other separate minor clades, without support among one another, were formed by species of subgroups Sitiens and Decens and the subgenus *Eumelanomyia*, with the genus *Lutzia* as a monophyletic clade (Figure 9).
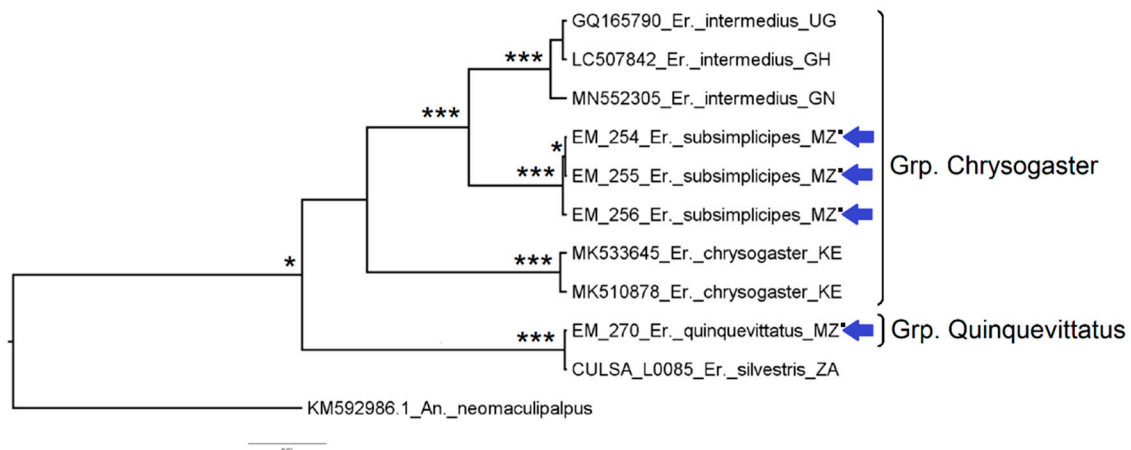
Figure 8. Phylogenetic analysis of 10 partial *coxI* nucleotide sequences from mosquitoes of the genus *Eretmapodites*. At specific branches, the number of * indicates the tree topological support revealed by the different phylogenetic reconstruction methods, assuming relevant bootstrap and aLRT values above 75% and posterior probability values above 0.80. The sequences obtained in this work are indicated with the "EM" code and those with associated genitalia are indicated by horizontal blue arrows; the "Grp" indicated group is marked by vertical lines. The sequences downloaded from GenBank and Boldsystems are indicated by their respective accession numbers and access codes (respectively); the symbol "." after the code of our sequence indicates that said sequence was not identified by Boldsystems. Their country of origin [South Africa (ZA), Ghana (GH), Guinea (GN), Mozambique (MZ), Kenya (KE), Uganda (UG)] are also indicated.
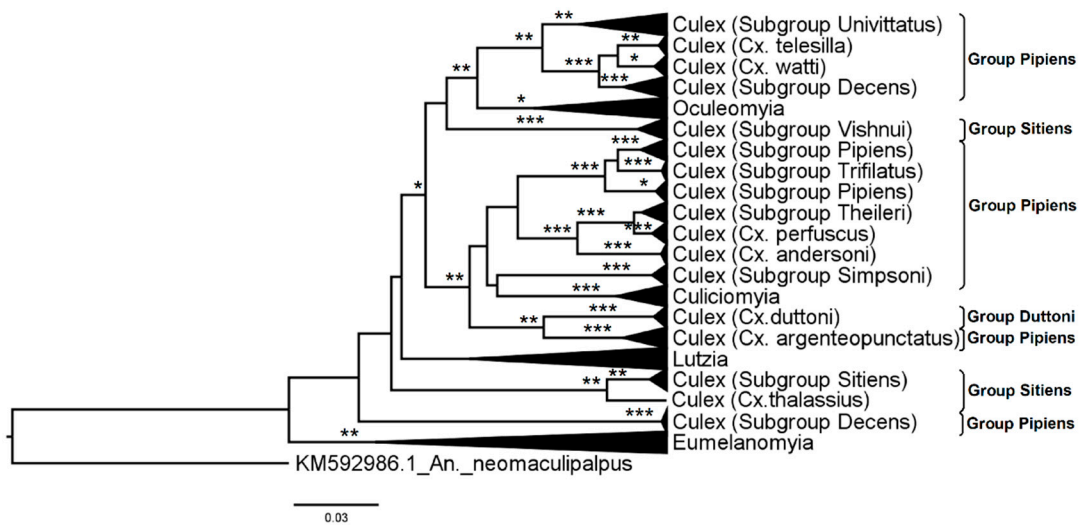

Figure 9. Phylogenetic analysis of 170 partial *coxI* nucleotide sequences from *Culex* and *Lutzia* mosquitoes. At specific branches, the number of * indicates the tree topological support revealed by the different phylogenetic reconstruction methods, assuming relevant bootstrap and aLRT values above 75% and posterior probability values above 0.80. In the collapsed branches there are species of the genus *Lutzia* and the subgenus and informal subgroups of the genus *Culex*; the vertical lines mark the informal groups.

The Univittatus subgroup formed a strongly supported clade, within which *Cx. (Cux.) univittatus* Theobald, 1901, *Cx. (Cux.) neavei* Theobald, 1906, and *Cx. (Cux.) perexiguus* Theobald, 1903, (File S-II Figures S27–S29) segregated into well-supported monophyletic clades (Figure 10). Sequences of *Cx. perexiguus* from South Africa and Mozambique clustered with sequences from other African countries, Europe, and the Middle East, with a divergence of 0.5% ± 0.2 between *Cx. perexiguus* from Europe and the Middle East and

those from Africa (File S-IV, Table S8). *Culex univittatus* from Africa were segregated from those of European origin. Networks and PCOORD analyses confirmed these results (File S-IV, Figure S11).
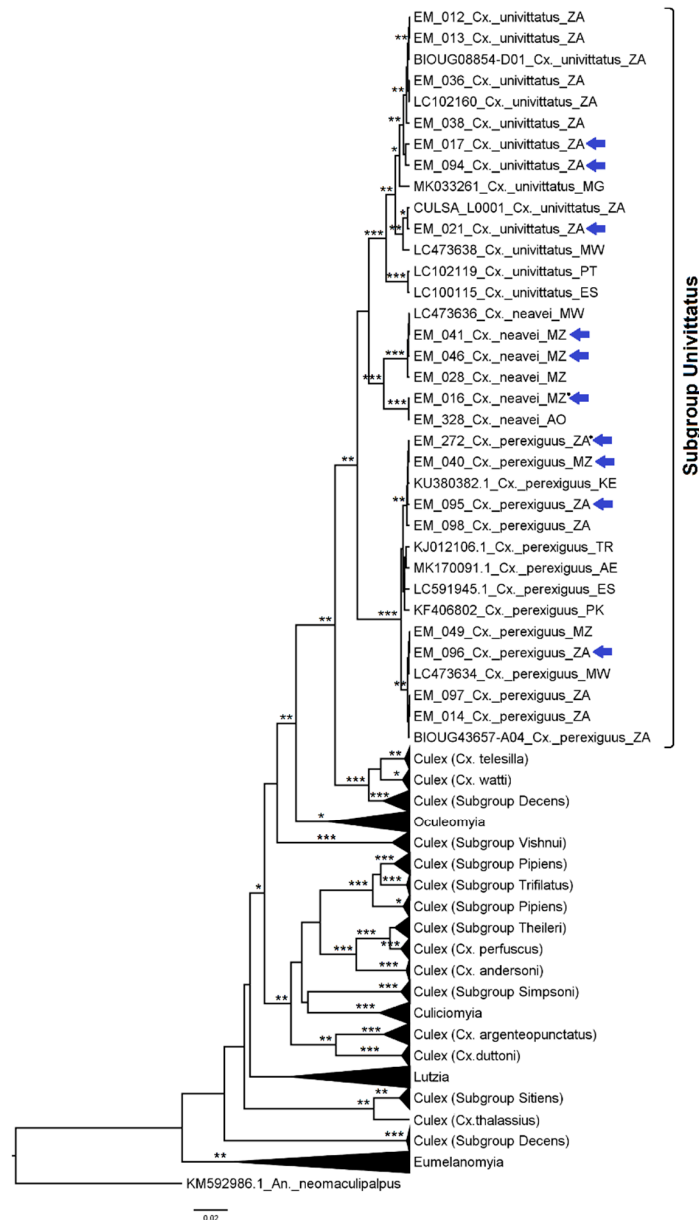

Figure 10. Phylogenetic analysis of 170 partial *coxI* nucleotide sequences from *Culex* and *Lutzia* mosquitoes, presenting the subgroup Univittatus. At specific branches, the number of * indicates the tree topological support revealed by the different phylogenetic reconstruction methods, assuming relevant bootstrap and aLRT values above 75% and posterior probability values above 0.80. The sequences obtained in this work are indicated by the "EM" code and those with associated genitalia are indicated by horizontal blue arrows. The sequences downloaded from GenBank and Boldsystems are indicated by their respective accession numbers and access codes (respectively); the symbol "." After the code of our sequence indicates that said sequence was not identified by Boldsystems. Their country of origin [South Africa (ZA), Angola (AO), United Arab Emirates (AE), Spain (ES), Madagascar (MG), Malawi (MW), Mozambique (MZ), Pakistan (PK), Portugal (PT), Kenya (KE), Turkey (TR)] are also indicated. The vertical line marks the subgroup Univittatus; the collapsed branches are the species of the genus *Lutzia* and subgenera and/or informal groups of the genus *Culex*.

Species of the pipiens complex, *Cx. quinquefasciatus*, and *Cx. pipiens* (File S-II Figures S31 and S32), and all those molecularly typed as *Cx. quinquefasciatus, Cx. pipiens* (*pipiens* ecotype plus hybrids of the *pipiens* and *molestus* ecotypes), formed a strongly supported clade (Figure 11). This monophyletic clade included *Cx. (Cux.) trifilatus* Edwards, 1914 (File S-II Figure S36), specimens of *Cx. pipiens* ecotype *molestus*, and one that could not be confirmed molecularly. The intra-clade variation supporting *Cx. quinquefasciatus, Cx. pipiens* (*pipiens* ecotype plus *pipiens-molestus* hybrids), and *Cx. trifilatus* was 1.6% ± 0.3, while the *molestus* ecotype diverged >2% in relation to the *pipiens* ecotype and *Cx. quinquefasciatus*, and *Cx. trifilatus* diverged ≥ 2.9% from any of the Pipiens subgroup members (File S-IV, Table S9). Similar results were obtained with networks and PCOORD analyses (File S-IV, Figure S12).
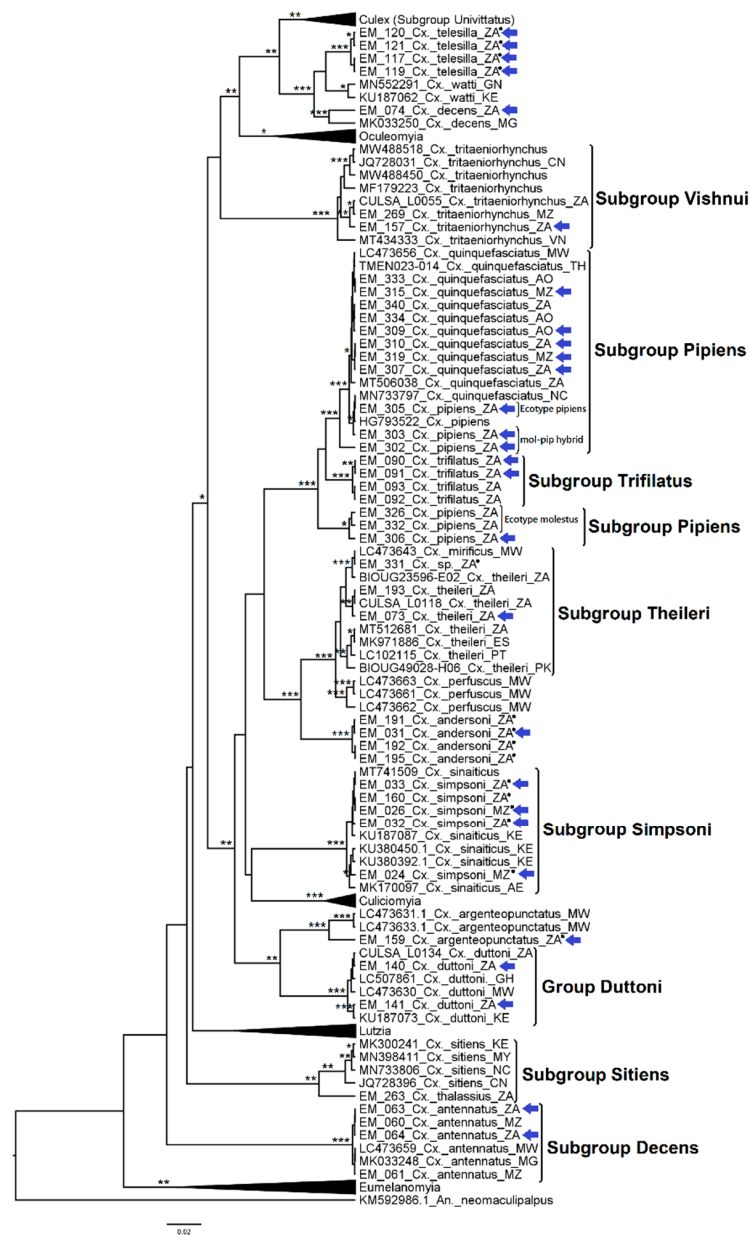

Figure 11. Phylogenetic analysis of 170 partial *coxI* nucleotide sequences from *Culex* and *Lutzia* mosquitoes, presenting the informal groups of the *Culex* subgenus. At specific branches, the number of * indicates the tree topological support revealed by the different phylogenetic reconstruction methods, assuming relevant bootstrap and aLRT values above 75% and posterior probability values

above 0.80. The sequences obtained in this work are indicated by the "EM" code and those with associated genitalia are indicated by horizontal blue arrows. The sequences downloaded from GenBank and Boldsystems are indicated by their respective accession numbers and access codes (respectively); the symbol "." after the code of our sequence indicates that said sequence was not identified by Boldsystems. Their country of origin [South Africa (ZA), Angola (AO), China (CN), Ghana (GH), Guinea (GN), United Arab Emirates (AE), Spain (ES), New Caledonia (NC), Madagascar (MG), Malaysia (MY), Malawi (MW), Mozambique (MZ), Pakistan (PK), Portugal (PT), Kenya (KE), Thailand (TH), Vietnam (VN)] are also indicated. The vertical lines mark the informal subgroups; the collapsed branches are the species of the genus Lutzia and subgenera and/or informal groups of the genus *Culex*, in addition to the ecotypes of *Cx. pipiens*.

Sequences from South African *Cx. (Cux.) theileri* Theobald, 1903 (File S-II Figure S35), another from a female originally identified (by us) as *Cx.* sp. (EM331) and *Cx. (Cux.) mirificus* Edwards, 1913 from Malawi (sharing 100% *CoxI* identity with EM331), formed sister clades with other sequences of *Cx. theileri* from Spain, Portugal, and Pakistan, with an intraspecific variation of 0.8% $\pm$ 0.2 (Figure 11). These were joined by *Cx. (Cux.) perfuscus* Edwards, 1914 and *Cx. andersoni* (File S-II Figure S24), forming a larger, well-supported monophyletic clade, a pattern that was supported by networks and PCOORD analyses (File S-IV, Figure S13).

Culex spp. of the subgroup Simpsoni formed a strongly supported clade in which the sequences of *Cx. (Cux.) simpsoni* Theobald, 1905 from this study, which had been morphologically confirmed through the male genitalia (File S-II Figure S33), did not segregate from the sequences of *Cx. (Cux.) sinaiticus* Kirkpatrick, 1925 from GenBank (Figure 11). Intraclade, intraspecific and interspecies divergence values overlapped, ranging from 0.2% to 0.4% ($\pm$0.1–0.2). These species were neither segregated by networks nor PCOORD analyses (File S-IV, Figure S14).

Subgenus *Oculeomyia* formed a monophyletic clade with branch support in only one of three methods (Figure 12); *Culex (Ocu.) bitaeniorhynchus* Giles, 1901 (File S-II File S44), *Cx. (Ocu.) infula* Theobald, 1901 (File S-II Figure S43), *Cx. (Ocu.) annulioris* Theobald, 1901 (File S-II Figure S41), and *Cx. (Ocu.) poicilipes* (Theobald, 1903) (File S-II Figure S42) sequences formed sister clades. However, the clades containing *Cx. bitaeniorhynchus* and *Cx. infula* were not species-specific; rather, sequences were grouped according to geographic origin, separating African specimens from ones originating in Asia. Hence, to unravel the relation of these taxa, we performed a further phylogenetic reconstruction with a larger data set (File S-IV, Figure S15). Similarly, African sequences obtained in this work deviated from the large clade formed by sequences from Asia and the Middle East, without separation of *Cx. bitaeniorhynchus* and *Cx. infula*. The distance between the various groups of sequences from the various countries of origin, or of different species, did not surpass 3%, and the divergence of these clades ranged between 2.0–2.7% (File S-IV, Table S10a,b). Networks and PCOORD analyses (File S-IV, Figure S16) still failed to separate *Cx bitaeniorhynchus* from *Cx. infula*.

The subgenus *Culiciomyia* was grouped into a defined clade with strong branch support, where *Cx. (Cui.) cinereus* Theobald, 1901 and *Cx. (Cui.) nebulosus* Theobald, 1901 (File S-II Figures S38 and S39) formed equally strong monophyletic clades (Figure 12) with low intraspecific variation for each branch ($\leq$0.4%), diverging by 3.6% $\pm$ 0.8.

Eumelanomyia sequences were grouped in an external clade of the remaining *Culex* subgenera (Figure 12), with *Cx. inconspicuosus* from South Africa (File S-II Figure S40) forming a strong clade with an intraspecific variation of 0.8% $\pm$ 0.3.
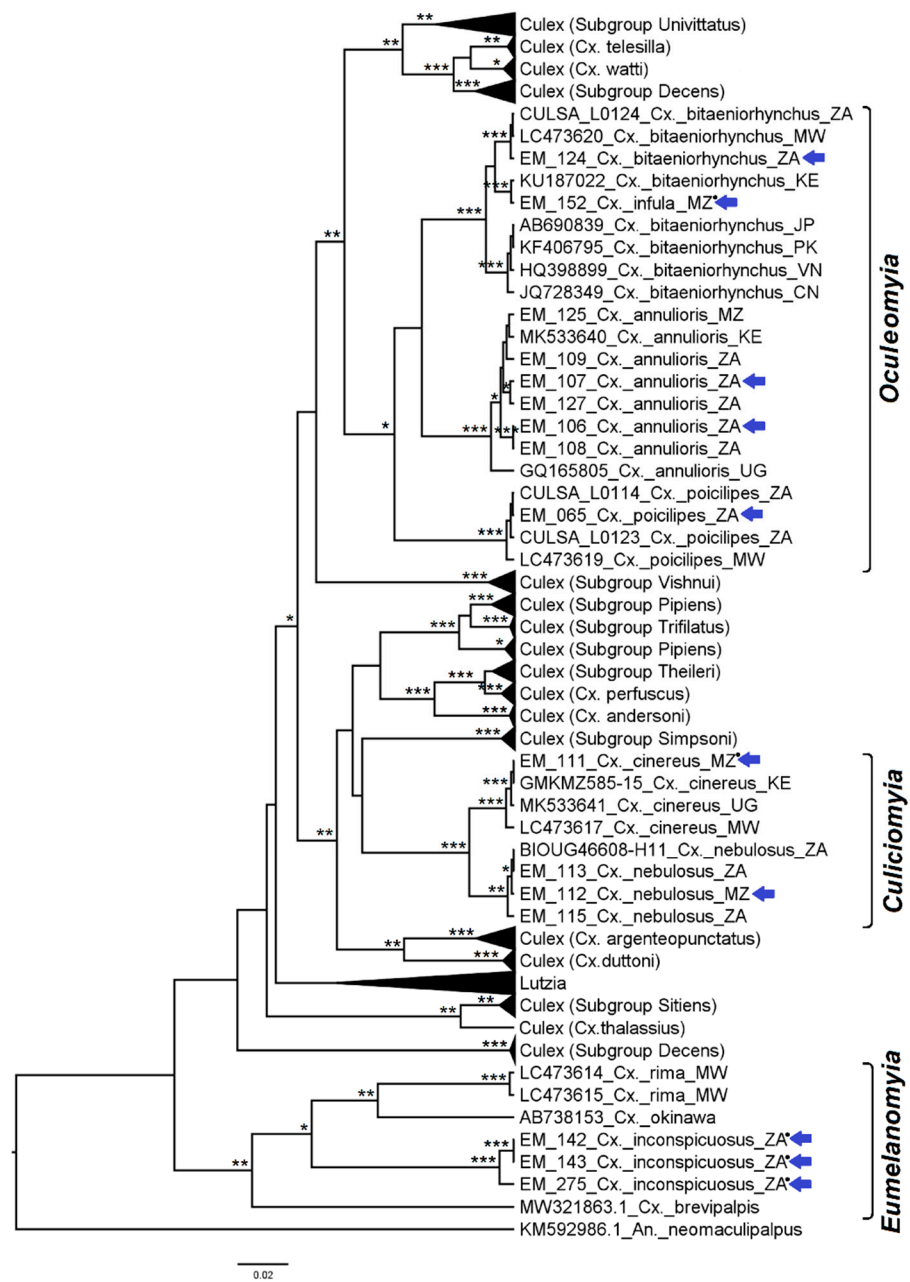
Figure 12. Phylogenetic analysis of 170 partial *coxI* nucleotide sequences from *Culex* and *Lutzia* mosquitoes, presenting the subgenera *Oculeomyia*, *Culiciomyia*, and *Eumelanomyia*. At specific branches, the number of * indicates the tree topological support revealed by the different phylogenetic reconstruction methods, assuming relevant bootstrap and aLRT values above 75% and posterior probability values above 0.80. The sequences obtained in this work are indicated by the "EM" code and those with associated genitalia are indicated by horizontal blue arrows. The sequences downloaded from GenBank and Boldsystems are indicated by their respective accession numbers and access codes (respectively); the symbol "." after the code of our sequence indicates that said sequence was not identified by Boldsystems. Their country of origin [South Africa (ZA), China (CN), Japan (JP), Malawi (MW), Mozambique (MZ), Pakistan (PK), Kenya (KE), Vietnam (VN), Uganda (UG)] are also indicated. The vertical lines mark the subgenera of *Culex*; the collapsed branches are the species of the genus *Lutzia* and informal groups of the genus *Culex*.

Sequences derived from *Lutzia (Metalutzia) tigripes* (de Grandpre & de Charmoy, 1901) from Angola and South Africa (File S-II Figure S45) were pooled with conspecific ones

from other African countries (Figure 13). When analyzing the relationship of the genus *Lutzia* with the other genera studied in this work, it grouped within a strongly supported clade that combined it with species of the subgenera *Culex*, *Oculeomyia,* and *Culiciomyia* (Figure 14).
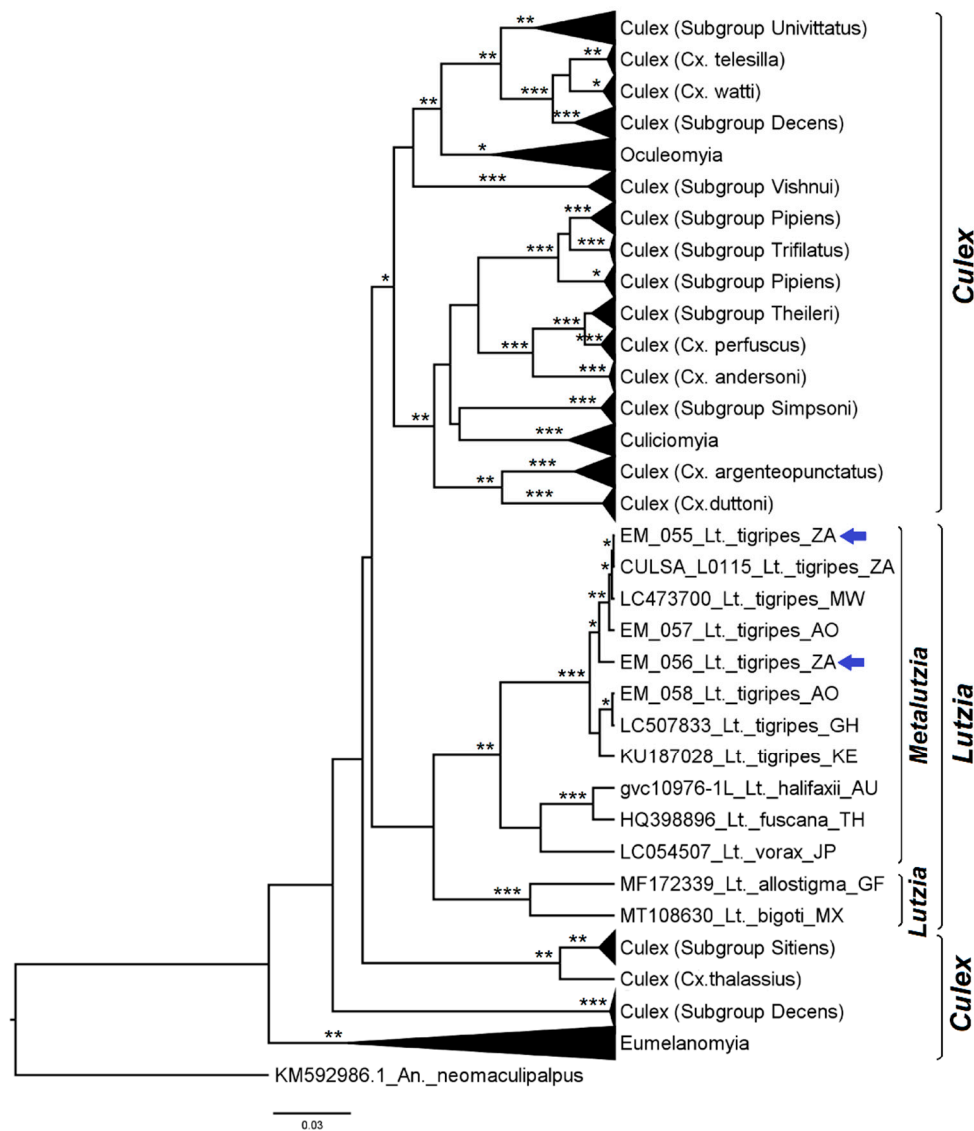

Figure 13. Phylogenetic analysis of 170 partial *coxI* nucleotide sequences from mosquitoes of the *Culex* and *Lutzia* genera, presenting the *Lutzia* genus. At specific branches, the number of * indicates the tree topological support revealed by the different phylogenetic reconstruction methods, assuming relevant bootstrap and aLRT values above 75% and posterior probability values above 0.80. The sequences obtained in this work are indicated by the "EM" code and those with associated genitalia are indicated by horizontal blue arrows. The sequences downloaded from GenBank and Boldsystems are indicated by their respective accession numbers and access codes (respectively). Their country of origin [South Africa (ZA), Angola (AO), Australia (AU), Ghana (GH), French Guiana (GF), Japan (JP), Malawi (MW), Mexico (MX), Kenya (KE), Thailand (TH)] are also indicated. The vertical lines mark the *Culex* genus and the *Lutzia* genus and its subgenera.
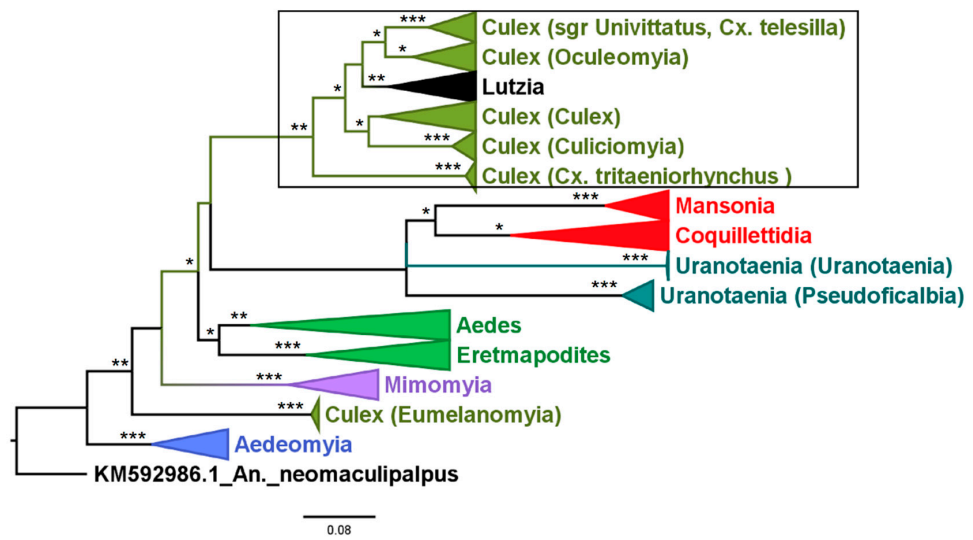
Figure 14. Phylogenetic analysis of 179 partial *coxI* nucleotide sequences from mosquitoes of the genera *Aedeomyia*, *Aedes*, *Coquillettidia*, *Culex*, *Lutzia*, *Mimomyia*, and *Uranotaenia*. At specific branches, the number of * indicates the tree topological support revealed by the different phylogenetic reconstruction methods, assuming relevant bootstrap and aLRT values above 75% and posterior probability values above 0.80. The collapsed branches indicate the different genera; "sgr" indicates the subgroup.

3.2.5. Genera *Ficalbia* and *Mimomyia*

The *CoxI* sequence obtained from *Fi. uniformis* (File S-II Figure S46) clustered inside the *Mimomyia* radiation, distant from the *Fi. minima* clade (Figure 15). Sequences of *Mi.* (*Mimomyia*) *mimomyiaformis* (Newstead, 1907) (File S-II Figure S47) and *Mi. (Mim) hispida* (File S-II Figure S48) clustered in a large clade, in which the former was organized into two strongly supported paraphyletic clades, with an overall intraspecific variation of 0.9% $\pm$ 0.3 (File S-IV, Table S11). Networks and PCOORD analyses revealed an identical pattern (File S-IV, Figure S17).
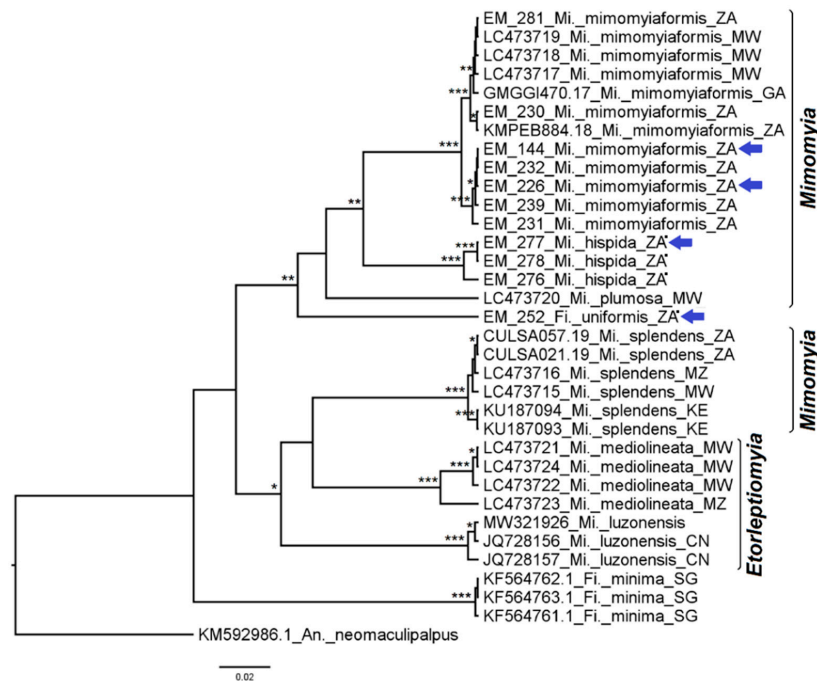

Figure 15. Phylogenetic analysis of 33 partial *coxI* nucleotide sequences from mosquitoes of the Ficalbia and Mimomyia genera. At specific branches, the number of * indicates the tree topological

support revealed by the different phylogenetic reconstruction methods, assuming relevant bootstrap and aLRT values above 75% and posterior probability values above 0.80. The sequences obtained in this work are indicated by the "EM" code and those with associated genitalia are indicated by horizontal blue arrows. The sequences downloaded from GenBank and Boldsystems are indicated by their respective accession numbers and access codes (respectively); the symbol "." after the code of our sequence indicates that said sequence was not identified by Boldsystems. Their country of origin [South Africa (ZA), China (CN), Gabon (GA), Malawi (MW), Mozambique (MZ), Kenya (KE)] are also indicated. The vertical lines mark the subgenera of Mimomyia and Etorleptiomyia.

3.2.6. Genus *Coquillettidia*

Sequences from the South African *Cq.* (*Coquillettidia*) *chrysosoma* (Edwards, 1915) specimens (File S-II Figure S51) grouped with *Cq.* (*Coq.*) *fuscopennata* (Theobald, 1903), *Cq.* (*Coq.*) *aurites* (Theobald, 1901), and *Cq. chrysosoma* sequences from Kenya, with an intra-clade variation of 0.2% ± 0.1 (Figure 16). Sequences of *Cq. fuscopennata* from South Africa (File S-II Figure S49) clustered with a sequence from Malawi in a monophyletic clade with an intraspecific variation of 0.5% ± 0.2, while another clade clustered GenBank sequences from *Cq. fuscopennata*, *Cq. (Coq.) versicolor* (Edwards, 1913) and *Cq. (Coq.) microannulata* (Theobald, 1911). The sequence of *Cq.* (*Coq.*) *metallica* (Theobald, 1901) from Mozambique (File S-II Figure S50) clustered in a monophyletic clade with an intraspecific variation of 1.1% ± 0.3. This was confirmed by network and PCOORD analyses (File S-IV, Figure S18).
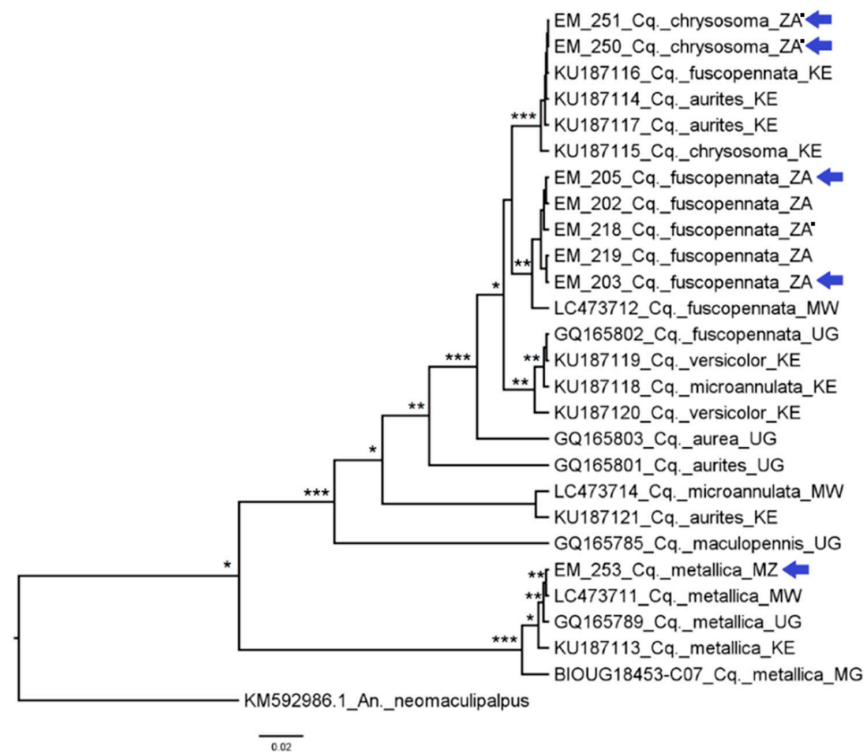

Figure 16. Phylogenetic analysis of 26 partial *coxI* nucleotide sequences from mosquitoes of the genus Coquillettidia. At specific branches, the number of * indicates the tree topological support revealed by the different phylogenetic reconstruction methods, assuming relevant bootstrap and aLRT values above 75% and posterior probability values above 0.80. The sequences obtained in this work are indicated by the "EM" code and those with associated genitalia are indicated by horizontal blue arrows. The sequences downloaded from GenBank and Boldsystems are indicated by their respective accession numbers and access codes (respectively); the symbol "." after the code of our sequence indicates that said sequence was not identified by Boldsystems. Their country of origin [South Africa (ZA), Madagascar (MG), Malawi (MW), Mozambique (MZ), Kenya (KE), Uganda (UG)] are also indicated.

3.2.7. Genus *Mansonia*

Mansonia (Mansonioides) africana (Theobald, 1901) and *Ma. (Mnd.) uniformis* (Theobald, 1901) were identified in this study based on female and male genitalia structures (File S-II Figures S52 and S53). Sequences of *Ma. uniformis*, from the Afrotropical and Indomalayan regions, were placed in two sister clades (Figure 17) with low intra-clade variation (ranging from 0.4 to 0.7%) but diverging from one another by 4.1% ± 0.9. *Mansonia africana* joined conspecific sequences from various African origins, with a divergence from *Ma. uniformis* ≥9%. These results were congruent with the network and PCOORD analyses (File S-IV, Figure S19).
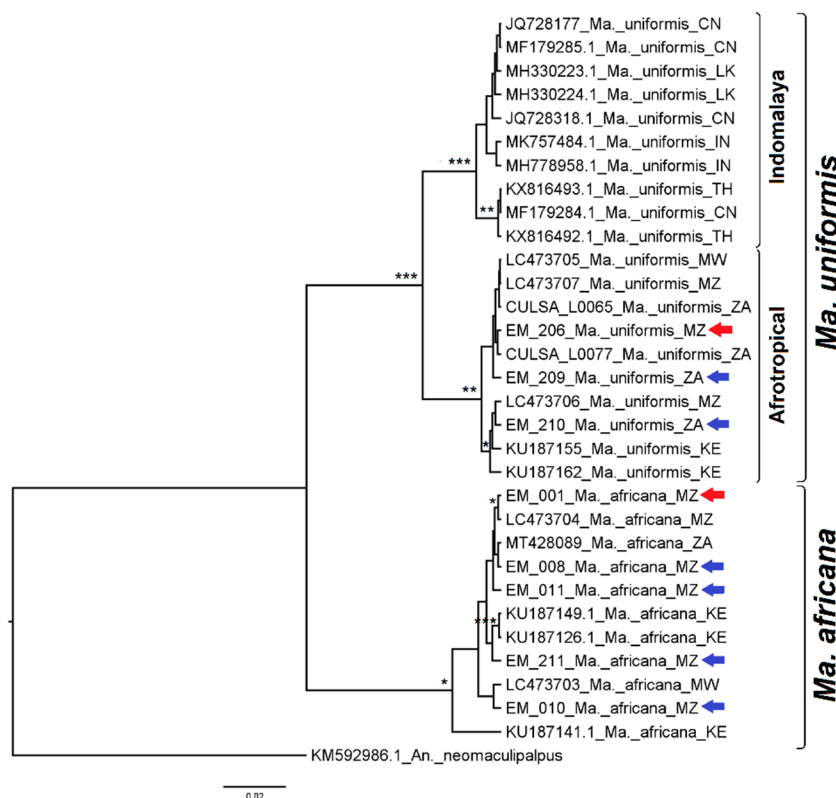

Figure 17. Phylogenetic analysis of 31 partial *coxI* nucleotide sequences from mosquitoes of the genus Mansonia. At specific branches, the number of * indicates the tree topological support revealed by the different phylogenetic reconstruction methods, assuming relevant bootstrap and aLRT values above 75% and posterior probability values above 0.80. The sequences obtained in this work are indicated by the "EM" code and those with associated genitalia are indicated by horizontal arrows (blue = males, red = females). The sequences downloaded from GenBank and Boldsystems are indicated by their respective accession numbers and access codes (respectively). Their country of origin [South Africa (ZA), China (CN), India (IN), Malawi (MW), Mozambique (MZ), Kenya (KE), Sri Lanka (LK), Thailand (TH)] are also indicated.

3.2.8. Genus *Uranotaenia*

Uranotaenia alboabdominalis (File S-II Figure S55) formed a strongly supported monophyletic clade (Figure 18) with an intraspecific variation of 0.2% ± 0.1. The sequences from *Ur. mashonaensis* (File S-II Figure S54) clustered into a monophyletic clade with strong support; however, the intraspecific variation was 4.1% ± 0.8, with a divergence of 5.6% ± 1.1 between the two branches.
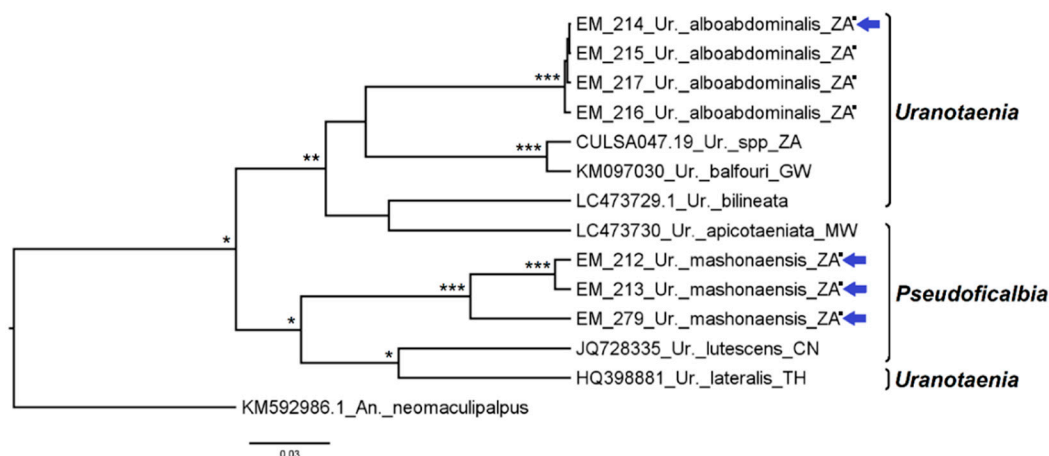
Figure 18. Phylogenetic analysis of 13 partial *coxI* nucleotide sequences from mosquitoes of the genus Uranotaenia. At specific branches, the number of * indicates the tree topological support revealed by the different phylogenetic reconstruction methods, assuming relevant bootstrap and aLRT values above 75% and posterior probability values above 0.80. The sequences obtained in this work are indicated by the "EM" code and those with associated genitalia are indicated by horizontal blue arrows. The sequences downloaded from GenBank and Boldsystems are indicated by their respective accession numbers and access codes (respectively); the symbol "." after the code of our sequence indicates that said sequence was not identified by Boldsystems. Their country of origin [South Africa (ZA), China (CN), Guinea-Bissau (GW), Malawi (MW), Thailand (TH)] are also indicated. The vertical lines mark the subgenera Uranotaenia and Pseudoficalbia.

4. Discussion

The genitalia of 663 mosquitoes (both male and female) were dissected and 55 species were identified; 247 partial sequences of the *CoxI* gene from 65 species were obtained and analyzed using complementary approaches, yielding a total of 67 species from 10 genera, identified by either method. This corresponded to *circa* 40% (60/150) of Culicinae mosquito fauna from South Africa and 34% (31/91) from Mozambique. Eleven of these partial *CoxI* sequences are, to the best of our knowledge, here published for the first time, with corresponding morphologic confirmation. Curiously, a considerable proportion of sequences that were generated failed to be identified either using the BOLD taxonomy tool (36%) or BLASTn (47%). In these cases, formal species assignment was carried out based on a fine morphological confirmation (genitalia) and/or by phylogenetic reconstruction.

Interspecific congeneric distances ranged between 1% and 20%, with mean values between 7% and 15% (Fiel S-IV Table S12). These values are mostly within the range observed for divergence in congeneric species, 2.3–21.8%, although the majority of cases are in the 4–11% interval (Ashfaq et al., 2014; Wang et al., 2012; refs. [21,27]). Low divergence values in congeneric taxa, such as 0.6–2.0%, can be interpreted as species of recent divergence [47]. Divergence between conspecific specimens typically ranges between 0% and 2.4% [21,27] or as high as 3% or 5.4% [18,48].

In some instances, divergence values > 2% in conspecific sequences were observed; hence, they were greater than expected in members of the same taxa; conversely, divergence values < 2% between taxa of different species were also obtained, revealing a failure of the *CoxI* marker to separate such taxa.

Higher than expected genetic diversity was observed in *Ad. africana*, where sequences from Malawi and Mozambique on the one hand and Kenya on the other formed separate clades with a divergence of 6.9%. *Aedes cumminsii* from South Africa fell within the Dentatus group as expected, jointly with *Ae. dentatus* from South Africa, a vector of RVFV and the Middelburg virus (MIDV) [2,3], while sequences from Kenya, Guinea, and Senegal formed a separate clade, diverging from the former by >7%. *Aedes unilineatus*, a monotypic member of the Unilineatus group [5] was considered a potential vector of ZIKV [49], with a very

wide distribution in sub-Saharan Africa, the Middle East, and Asia [1], in which South African sequences and those originally from Pakistan had an interclade variation of 5.3%. *Aedes metallicus,* the monotypic member of the Metallicus group [5], an important vector of sylvatic YFV and potential vector of ZIKV in Africa [2], had high sequence divergence of 7.4% between South Africa and Mozambique versus that from Kenyan specimens. *Aedes furcifer* from South Africa, Kenya, and Senegal, had an intraspecific variation of 8.1%. *Aedes caballus* from South Africa clustered separately from those originating in Iran, with a divergence of ≥3.6% between them. *Mansonia uniformis* formed separate clades, according to African or Indomalayan-Asian origin, with a divergence of 4% between them. *Uranotaenia mashonaensis* from South Africa had a considerable variation of 4.1%.

Such divergence may be explained, in some cases, by comparing sequences of naturally different conspecific populations collected far apart geographically, such as in this study with *Ae. unilineatus*, *Ae. caballus*, and *Ma. uniformis*, which compared sequences of specimens originating from South Africa to those from the Asian region. However, others such as *Ad. africana*, *Ae. cumminsii*, *Ae. metallicus*, *Ae. furcifer*, and *Ur. mashonaensis* exhibited a large genetical divergence in sequences between specimens originating from a span of regional context in the African continent, e.g., South Africa, Mozambique, and Kenya.

It is not surprising that *Aedes cumminsii*, a vector of MIDV, Spondweni virus (SPOV), and RVFV [2], had considerable within-species sequence variations, as it is likely a sibling species complex. Throughout its broad savanna- and forest-dwelling distribution in Africa, many morphological variations have been noted (AJC personal communication and [4]). In addition, *Ae. cumminsii* has undergone some taxonomic confusion since it was originally described as a now designated valid subspecies, ssp *mesostictus* [1], which was originally named ssp *mediopunctatus* (Theobald, 1909) and later placed synonymously and elevated to a subspecies of *Ae. cumminsii* [50]. This subspecies was originally described from specimens collected in Ghana and differs from the typical form of *Ae. cumminsii* by the presence of small basal median whitish spots on the abdominal tergites in both sexes [4,5]; however, McIntosh [46] suggested that this subspecies occurs only in southern Africa. We identified *Ae. cumminsii* with the typical features of the subspecies *mesostictus* in northeastern South Africa, such as Guarido et al. [12], with a divergence of >7% from specimens from Kenya [15].

A lack of CoxI sequence separation of taxa of different species was found between *Ad. madagascarica* and *Ad. africana*; *Ae. mcintoshi*, *Ae. circumluteolus*, and *Ae. unidentatus*; *Ae. simpsoni* and *Ae. bromeliae*; *Ae. scatophagoides* and *Ae. sudanensis*; *Cx. quinquefasciatus* and *Cx. pipiens*; *Cx. simpsoni* and *Cx. sinaiticus*; and *Cx. bitaeniorhynchus* and *Cx. infula*. In these cases, they could not be correctly segregated into species-specific clusters either by traditional phylogenetic reconstruction, networks, or PCOORD analyses. Fortunately, although being morphologically similar species, they can still be differentiated by fine morphological details or male genitalia. Furthermore, genetic distance analysis disclosed overlapping intra- and inter-specific values, circa <2%, showing a limitation in their resolution capacity. Such overlap has been responsible for misidentifications and impossibilities of delimiting species based on pairwise distances [17,51]. One such example includes the segregation of *Ad. africana* from Mozambique (EM_245, LC662529) and Malawi (LC473725) with *Ad. madagascarica* (MK033247.1). Although *Ad. madagascarica* has only been described in Madagascar, the genetic divergence between this species and Ad. africana was only 0.2%. In a contrasting situation, *Ad. africana* CoxI sequences from neighboring countries were separated as aforementioned, raising the need for further clarification of the significance of both the similarity between sequences of *Ad. madagascarica* and *Ad. africana* from Mozambique and Malawi and the divergence of *Ad. africana* from Mozambique and Malawi versus Kenya.

Among the *Aedes*, the subgenus *Neomelaniconion* includes potential vectors of arboviruses, such as *Ae. mcintoshi*, a major vector of RVFV, and *Ae. circumluteolus* and *Ae. unidentatus* as potential RVFV vectors [2], but also potential vectors of the Shuni virus (SHUV) [31]. These three taxa could not be differentiated through phylogenetic reconstruc-

tion, in agreement with previous findings [12,13]. Evidence from Kenya suggests that *Ae. mcintoshi* forms a complex of morphologically indistinguishable species, with discordant results between *CoxI* and *ITS* markers [38], particularly *ITS2*, thereby failing to resolve species and species complexes in the subgenus *Neomelaniconion* in Madagascar [52].

Culex pipiens and *Cx. quinquefasciatus*, which are members of the *Culex pipiens* complex, not only display wide geographic distribution but are also highly relevant in the transmission of various pathogens, including arboviruses such as USUV, WNV, and SINV [1]. The female specimens of the two species are morphologically similar but differ in their vectorial efficiency and may occur sympatrically; additionally, hybridization has been reported in some locations [53–55], but not in South Africa [53]. In this study, the absence of *pipiens-quinquefasciatus* hybrids in southern Africa was also noted. Hybrids of the *molestus* and *pipiens* ecotypes, which, so far, have only been reported in the United States [56], Southern Europe [55], and North Africa [57], have also been described in this work as male *Cx. pipiens* specimens from South Africa. Curiously, while *CoxI* analyses could not resolve the closely convergent *Cx. quinquefasciatus* and *Cx. pipiens* into species-specific clusters, the *molestus* ecotype sequences clustered out, diverging 2.2–2.8% in relation to *pipiens* ecotype, *pipiens-molestus* hybrids, and *Cx. quinquefasciatus*. Other studies have reported an intraspecific variation in *Cx. pipiens* (3%), larger than the interspecific distance with *Cx. quinquefasciatus* (1.6%) and their lack of separation [18,24]. The *CoxI* gene has been successful in differentiating members of the *Cx. pipiens* complex, though with low variability; therefore, it may not be the better marker to infer the evolutionary relationship of such close taxa, and more polymorphic markers or a multilocus analysis may be more informative [58]. A lack of differences in *CoxI* between different species can also be explained by possible introgression of mitochondrial DNA after several interspecific crosses, which was proven for *Culex* species using several DNA markers [58].

Culex bitaeniorhynchus and *Cx. infula* belong to the *Cx. bitaeniorhynchus* complex of the subgenus *Oculeomyia*; *Cx. bitaeniorhynchus* has a wide distribution, being present in tropical and subtropical areas of the Afrotropical, Southern Palearctic, and Indomalayan regions, and on the mainland and islands of Southeast Asia and Australasia [1], and can be involved in the transmission of arboviruses [34]. Its status, as well as that of *Cx. infula* and *Cx. ethiopicus* Edwards, 1941, has been the subject of controversy [34,36]. The morphological characteristics of the specimens identified as *Cx. bitaeniorhynchus* (synonymous *Cx. ethiopicus*) corresponded to those described by Edwards [4] and Jupp [5], while the specimen of *Cx. infula* from Mozambique only allowed us to ascertain it as a *Cx.* sp., confirmed by the analysis of the genitalia. Malawian mosquitoes identified as *Cx. ethiopicus* were found to differ in the shape of the wing scales and diverge in the *CoxI* gene > 2% with *Cx. bitaeniorhynchus* from Asia [13]. In our study, the genetic divergence ranged from 2.0% (±0.6) to 2.7% (±0.7) with sequences from Asia, and the tree topology, networks, and PCOORD analysis suggest that *CoxI* does not have discriminating power for separating *Cx. bitaeniorhynchus* from *Cx. infula*. The *Cx. infula CoxI* sequence from Mozambique also deviated from *Cx. infula* from Asia by a similar range. So far, *Cx. infula* has only been described in Asia [1]; however, we were able to confirm its previous identification by Ribeiro in Angola of five male specimens which he designated as *Cx. bitaeniorhynchus* [59]. Furthermore, specimens collected in Africa continue to be classified as *Cx. bitaeniorhynchus* and *Cx. ethiopicus*, according to Edwards' [4] nomenclature [13,29]. Altogether, based on the evidence presented, we believe that most of the specimens identified as *Cx. bitaeniorhynchus* in Africa actually are *Cx. infula*, a situation that Harbach [34] had already anticipated.

Aedes aegypti Linnaeus, 1762 is one of the most important vectors of several global health impact arboviruses, such as DENV, ZIKV, CHIKV, and YFV, not only in Africa but globally [1,2,60]. In the phylogenetic analysis, all sequences of *Ae. aegypti* grouped into a single clade with strong branch support. Although this clade was divided into two branches, albeit only one with reasonable support, and both with a very small distance, there were no data to support the notion that these may correspond to either subspecies *Ae. aegypti aegypti* or *Ae. aegypti formosus* Walker, 1848. Our samples and data set were not

adequate for such a separation as no clear morphological differentiation was noticed in our specimens. A small set of sequences (N = 10) was analyzed, and for low variability data, as in this case, the phylogenetic study performed was not that indicated, and haplotype analyses of markers such as mtDNA ND4 [61] or microsatellites [62] were necessary.

As to the arrangement of genera, on the whole, *Aedes* sequences formed clusters that mainly corresponded to the subgenera, in agreement with the morphology-based taxonomy, including the informal groups proposed by McIntosh for the species of Subgenus *Aedimorphus* [5,46]. Subgenera *Neomelaniconion*, *Stegomyia*, *Catageiomyia*, *Fredwardsius*, *Ochlerotatus*, and *Mucidus* formed monophyletic clades, but *Diceromyia*, *Aedimorphus*, and *Albuginosus* did not. Subgenus *Diceromyia* was represented by *Ae. furcifer* and *Ae. fascipalpis*, which, although well separated, yielded a paraphyletic arrangement. Genus *Aedes* is a highly complex entity, the taxonomy of which is in dire need of clarification [35], and that mitogenome evolutionary analysis has shown to be paraphyletic [63].

Genus *Culex* segregated into a highly paraphyletic topology, where most species of subgenus *Culex* coincided with the informal groups and subgroups proposed by Harbach [64]; however, mitogenome phylogenetics has found genus *Culex* to be monophyletic [63]. To the best of our knowledge, this is the first phylogenetic study including African members of the subgenus *Culiciomyia*. While sequences of only two species were included in this study, the monophyly of *Culiciomyia* was well supported, and the *CoxI* marker performed well in the discrimination of *Cx. nebulosus* and *Cx. cinereus*. Multiple species of *Culiciomyia* occur in Africa and all have identical female and male external morphologies, relying solely on male genitalia structures for species identification [65]. Separation of species by *CoxI* gene sequences may be fruitful in the case of *Culiciomyia*.

Lutzia tigripes is the only representative of the genus *Lutzia* in the Afrotropical region [1], clustering as a monophyletic assemblage within the *Culex* radiation, as previously found with *CoxI* and *ITS2* [20,22,66]. Morphological data from adults and larvae support different patterns of relationships between *Lutzia* and *Culex* [67], while a recent analysis of the complete mitochondrial genome concluded that *Lutzia* forms a monophyletic group with genus status [68], emphasizing the limitations of phylogenetic studies with a single marker. However, the classification controversy is not limited to the genus *Lutzia*. Support for the monophyly of Culicini generic-level groups is granted for all except subgenera *Culex*, *Eumelanomyia*, and *Neoculex* [67]. Our analysis could not confirm subgenus *Culex* as a monophyletic group, while *Eumelanomyia* formed a clade distant from the remaining *Culex*, in agreement with previous works [23,28,69]. Nevertheless, the study of all genera together yielded some interesting results; except for subgenus *Eumelanomyia* of *Culex*, there was support for tribes Aedini, Culicini, Ficalbiini, and Mansoniini, in agreement with the monophyly of genera *Mansonia*, *Coquillettidia*, and *Culex*, through mitochondrial phylogenomics [63].

In most of these cases, representative studies involving more taxa, a higher number of specimens per taxa sampled over a wider geographic range, and merging morphological and molecular characterization are needed to unravel the specific status of different populations and characterize species complexes in Africa and their relationship with their members elsewhere and/or the monophyly/paraphyly of some subgenera or genera. The systematics within the Culicini tribe cannot be resolved with morphological data alone [67], stressing the relevance of obtaining new molecular data.

Circa 40% (36–47%) of the sequences obtained in this study could not be correctly identified using BOLD and BLASTn as identification tools; this was because (i) the sequence was obtained for the first time, (ii) they had been obtained from members of species complexes, or (iii) there was an incorrect assignment, including at the genus level, such as sequences from *Cx. inconspicuosus* and *Ae. durbanensis* that were identified in BOLD as *Ae. argenteopuntatus* and *Cx. tritaeniorhynchus*, respectively, with >99% probability. Although studies that associate morphological and *CoxI* barcode-based molecular identifications are increasing, few include a definite diagnostic identification [20,24,48]. This absence is a potential source of error, as many species are only distinguishable by subtle morphological

differences in the male genitalia. In fact, we have detected sequences from the barcode fragment of the *CoxI* gene from GenBank and Boldsystems which, given their phylogenetic signal, suggested the possibility of misassignment to another species. Examples of such are the *Coquillettidia* heterospecific sequences that clustered with our sequence of *Cq. chrysosoma* and the sequence ascribed to *Er. Silvestris*, which had complete identity with our sequence of *Er. quinquevittatus*, given that in both cases we had the morphology-based identification to the level of male genitalia for our sequences. This type of error has already been identified in other studies based on the *CoxI* marker [25,26] and *ITS* [33]. Such species assignment errors are perpetuated and amplified when authors consider only genetic similarities with previous GenBank entries. *CoxI*-based barcoding should complement morphologically-based identification [20], rather than species identification being based only on genetic similarities with existing sequences in the GenBank database [15].

Incorrect assignments also cause irregular situations in the BINs (barcode index numbers) assigned to what the BOLD system defines as operational taxonomic units, ideally corresponding to different species. For example, as in the case of the *Aedeomyia* species from the Afrotropical region, where three BINs were identified, the first for *Ad. furfurea* from Malawi (BOLD:AEH5592), the second BIN was shared between *Ad. madagascarica* and *Ad. africana* (BOLD:ADV5603) and the third was shared between *Ad. africana* and *Ad. furfurea* (BOLD:ACK8488). In the third case, there may have been an incorrect assignment of certain sequence(s) to the species *Ad. furfurea*, a situation that phylogenetic analysis was able to resolve. In other cases, more than one species clustering into one BIN have been registered, and another species has been split into more than one BIN [26].

5. Conclusions

Our study has contributed to the barcode library of Afrotropical mosquitoes, some of which are known potential vectors of arboviruses [2,3] or have recently been found to be so, or carriers of insect specific flavivirus [30–32,70]. This was achieved by associating careful morphologically identified referenced voucher specimens to specific molecular marker *CoxI* partial sequences. However, partial *CoxI* sequences have been shown to fail in unambiguously discriminating some proximal species or members of species complexes in addition to overestimating the diversity of *Culex* spp. [17]. Hence, it will be necessary to use alternative molecular markers, including nuclear, such as *Ace2* [11], microsatellites [54–56], or mitochondrial, such as *ITS*, to molecularly delineate species. However, that may prove to not always be sufficient [52] and other markers such as 16S [26], ND4 [71], or the complete mitochondrial genome [63,68] may be required.

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