MOLECULAR PHYLOGENY OF SOUTH AFRICAN *ANOPHELES*, *AEDES* AND *CULEX* (DIPTERA: CULICIDAE) BASED ON COI, ITS2 AND 28S DNA SEQUENCES

by

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Submitted in fulfilment of the requirements for the degree

MAGISTER SCIENTIAE

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November 2021

DECLARATION

I, Liezl Whitehead, declare that the Master's research dissertation that I herewith submit at the University of the Free State, is my independent work and that I have not previously submitted it for qualification at another institution of higher education.

November 2021

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ABSTRACT

Mosquitoes have a substantial impact on human and animal health as vectors of disease and consequently take a heavy toll on the economy. South Africa hosts a multitude of mosquito-borne pathogens, including numerous arboviruses and *Plasmodium.* Many pathogens are transmitted by species currently belonging to three genera, namely *Aedes* Meigen, 1818, *Culex* Linnaeus, 1758 and *Anopheles* Meigen, 1818. Mosquitoes have traditionally been classified by their morphology, which has produced several non-monophyletic taxa. However, the field of systematics has been revolutionised with the advent of molecular techniques, which allow researchers to gain novel insights into the hidden affiliations of taxa. Since few phylogenetic studies have focused on South African mosquitoes, the current study aimed to investigate the intrageneric relationships of South African *Aedes, Culex* and *Anopheles* species based on COI, ITS2 and 28S DNA sequences. Therefore, mosquitoes were sampled across various habitats within central South Africa, ranging from relatively pristine nature reserves to urban centres with substantial anthropological activity. Morphological variations of sampled specimens were noted and numerous morphotypes were sequenced for the three target DNA regions. The identities of sampled specimens were verified with BLAST queries and the generated sequences were incorporated into genus-specific multiple sequence alignments. The datasets included representatives of many major taxonomic subdivisions and all accessible South African species, which were obtained from international DNA repositories. Due to the limited availability of sequences, it was not possible to construct concatenated datasets consisting of all three target DNA regions. Therefore, the aim was rather to incorporate shared representative species within each of the independent COI, ITS2 and 28S datasets. These genus and gene-specific multiple sequence alignments were used to conduct maximum likelihood and Bayesian phylogenetic analyses. Several phylogenetic findings were corroborated by multiple gene regions and supported by the results of other authors. This included the polyphyly of several *Aedes* subgenera (*Aedimorphus*, *Ochlerotatus* and *Stegomyia*) and the non-monophyly of several *Neomelaniconion* species. The subgenus *Culex* was also often rendered non-monophyletic by two other subgenera (*Barraudius* and *Oculeomyia*) and similarly contained polyphyletic assemblages of *Cx. pipiens, Cx. quinquefasciatus* and the Pipiens Group. Finally, the current analyses also recovered non-monophyletic groupings of the subgenus *Anopheles*, the Laticorn Section and the Funestus Group within the genus *Anopheles*. These results emphasized consistent phylogenetic findings, revealing numerous challenges within the current systematic framework. Therefore, the study provided insight into the affiliations of numerous South African species, generated molecular and phylogenetic data for sampled individuals and provided distribution data for the associated specimens. These results expanded the foundation of phylogenetic and ecological data, which can be incorporated into other epidemiological, biogeographical and evolutionary investigations.

Keywords: South Africa, *Aedes*, *Culex*, *Anopheles*, molecular phylogeny.

ACKNOWLEDGEMENTS

My appreciation of entomology and genetics was guided, shaped and refined by a community of passionate individuals who generously imparted some of the knowledge that they have accumulated over many years of experience. I would like to express my sincere appreciation to all individuals who contributed to the final outcome of this project.

I am thankful to each of my supervisors and co-supervisors for their unique contributions to the project, where their fields of expertise coalesced to expand my knowledge, experience and growth as a researcher.

To my supervisor Dr Vaughn Swart, thank for your continuous patience, guidance and support. You fashioned the foundation for this project and provided me with ample opportunities to broaden my knowledge and experience the remarkable entomological community. To Dr Marieka Gryzenhout, thank you for your guidance as my molecular mentor and for the opportunity to gain hands-on experience with the wonders of genetics. To Prof Lizette Koekemoer, thank you for your valued advice on the technicalities of mosquito research and the refinement of the project.

Thank you to Ruan Booysen and my parents, Charles and Marietjie Whitehead, for your greatly appreciated assistance during my sampling efforts. Thank you to Alan Kemp, who familiarised me with some of the practical aspects of mosquito research, and Thabang Madisha who provided ample guidance with the molecular aspects of this project. I would also like to thank Stephanus Riekert who took the time to provide details on the use of the university's high performance computing system.

This project would not have been possible without the generous financial contributions of Prof Felicity Burt, Dr Marieka Gryzenhout, Dr Vaughn Swart and the Grassland Biodiversity Project, which provided funding for the necessary reagents, consumables and sampling efforts. Furthermore, I would like to thank the UFS and the NRF for the academic and personal financial assistance during my Master's research (NRF reference no: SFH170609238850).

Finally, I would like to express my sincere appreciation towards my family and friends for their unwavering support throughout my postgraduate journey. You have been the foundational pillars in my life and have always supported me in my entomological pursuits.

LIST OF ACRONYMS

Acronym	Meaning
28S	large subunit ribosomal RNA
Acy.	subgenus *Acartomyia*
Adm.	subgenus *Aedimorphus*
Ae.	genus *Aedes*
Aed.	subgenus *Aedes*
All.	subgenus *Allotheobaldia*
An.	genus *Anopheles*
Ano.	subgenus *Anopheles*
Arm.	subgenus *Armigeres*
BANV	Banzi virus
Bar.	subgenus *Barraudius*
BLAST	Basic Local Alignment Search Tool
BOLD	Barcode Of Life Data System
bp	base pair
BS	bootstrap support
BUNV	Bunyamwera virus
Cat.	subgenus *Catageiomyia*
CDC	Centers for Disease Control
Cel.	subgenus *Cellia*
CHIKV	Chikungunya virus
CO_2	carbon dioxide
Coe.	subgenus *Coetzeemyia*
COI	cytochrome c oxidase subunit I
Cs.	genus *Culiseta*
Cui.	subgenus *Culiciomyia*
Cux.	subgenus *Culex*
Cx.	genus *Culex*
D1	domain 1
Dah.	subgenus *Dahliana*
DENV	Dengue virus
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
Dob.	subgenus *Dobrotworskyius*
Dow.	subgenus *Downsiomyia*
Eum.	subgenus *Eumelanomyia*
GC	Group present / Contradicted
Grg.	subgenus *Georgecraigius*
GTR	*general time* reversible
How.	subgenus *Howardina*
Hul.	subgenus *Hulecoeteomyia*
ITS1	first internal transcribed spacer
ITS2	second internal transcribed spacer
Jar.	subgenus *Jarnellius*
Ker.	subgenus *Kerteszia*
LMS	lower mesanepimeral setae
Lop.	subgenus *Lophoceraomyia*
Ma.	genus *Mansonia*
Mac.	subgenus *Macleaya*
Mai.	subgenus *Maillotia*
Mcx.	subgenus *Microculex*
Mel.	subgenus *Melanoconion*
$MgCl_2$	magnesium chloride
MIDV	Middelburg virus
ML	maximum likelihood
Mnd.	subgenus *Mansonioides*
MP	maximum parsimony
MPSAM	Molecular Phylogeny of South African Mosquitoes
MSA	multiple sequence alignment
mtDNA	mitochondrial DNA
Muc.	subgenus *Mucidus*
Ncx.	subgenus *Neoculex*
ND4	nicotinamide adenine dinucleotide hydride dehydrogenase subunit 4
NDUV	Ndumu virus
Neo.	subgenus *Neomelaniconion*
NJ	neighbour-joining
NRF	National Research Foundation
NRIV	Ngari virus
Och.	subgenus *Ochlerotatus*
Ocu.	subgenus *Oculeomyia*
ORUV	Orungo virus
PCR	polymerase chain reaction
PGAV	Pongola virus
Phc.	subgenus *Phenacomyia*
PI	percentage identity
Pmt.	subgenus *Polyleptiomyia*
PP	posterior probabilities
Pro.	subgenus *Protomacleaya*
Psk.	subgenus *Pseudoskusea*
Ram.	subgenus *Rampamyia*
rDNA	ribosomal DNA
RNA	ribonucleic acid
Rus.	subgenus *Rusticoidus*
RVF	Rift Valley fever
SFV	Semliki Forest virus
SINV	Sindbis virus
SNP	single nucleotide polymorphisms
SPONV	Spondweni virus
Ste.	subgenus *Stethomyia*
Stg.	subgenus *Stegomyia*
Tan.	subgenus *Tanakaius*
UFS	University of the Free State
UPGMA	unweighted pair group method with arithmetic mean
USUV	Usutu virus
WSL	Wesselsbron virus
WNV	West Nile virus
YFV	Yellow Fever virus
Zav.	subgenus *Zavortinkius*

CHAPTER 1 – INTRODUCTION

1.1 SIGNIFICANCE OF MOSQUITOES

1.1.1 MOSQUITOES AS VECTORS OF DISEASE

Mosquitoes are prominent vectors of disease and have a substantial impact on human and animal health. Their blood-feeding behaviour serves as an opportunity for the transmission of pathogens, including viruses, filarial worms and protozoans (Harbach 2007). These mosquito-borne diseases result in millions of human fatalities annually (World Health Organization 2020), consequently taking a heavy toll on the economy (Gallup & Sachs 2001). The agricultural sector is also burdened by the transmission of numerous livestock diseases (Jupp 1996). This impact is especially relevant within Africa's developing nations, since many of the challenges associated with poverty can exacerbate the occurrence of mosquito-borne diseases (Teklehaimanot & Mejia 2008).

Africa has a relatively high diversity of mosquito-borne arboviruses (Braack *et al*. 2018), with many occurring within South Africa. The viruses relevant to South Africa include the Bunyamwera virus, Bwamba virus, Semliki Forest virus (Kokernot *et al.* 1956), Banzi virus, Germiston virus, Witwatersrand virus (McIntosh *et al*. 1976), Lumbo virus (Kokernot *et al*. 1962), Middelburg virus (Van Niekerk *et al*. 2015), Ndumu virus (Kokernot *et al*. 1961), Pongola virus (McIntosh *et al*. 1972), Rift Valley fever virus (Jupp 1996), Shuni virus (Van Eeden *et al.* 2012), Spondweni virus (McIntosh *et al.* 1961), Sindbis virus, Wesselsbron virus, West Nile virus (Weiss *et al*. 1956, Weyer *et al*. 2013), Usutu virus (Engel *et al*. 2016) and infrequent epidemics of dengue and chikungunya (McIntosh *et al*. 1977, Amarasinghe *et al*. 2011). In addition to arboviruses, South Africa also sustains the transmission of the malaria protozoan, *Plasmodium* (Sharp *et al*. 2007) and has experienced individual cases of dirofilariasis in humans and animals (Verster *et al.* 1991, Moodley *et al*. 2015). Many of these pathogens result in human illness, while several of these viruses also affect domestic ungulates (Table 1.1).

These mosquito-borne diseases are mainly transmitted by mosquitoes currently classified as members of the genera *Anopheles* Meigen, 1818, *Aedes* Meigen, 1818, and *Culex* Linnaeus, 1758. Several species within the genus *Aedes* transmit medically and veterinary important viruses in South Africa, including chikungunya, dengue fever and Rift Valley fever (Prinsloo 2006). Certain South African *Culex* species also serve as vectors of Rift Valley fever, Sindbis and West Nile fever (Prinsloo 2006). Finally, one of the most medically relevant pathogens, *Plasmodium* Marchiafava & Celli, 1885, is transmitted by several *Anopheles* species (Sharp *et al.* 2007).

Table 1.1. Pathogens of medical and veterinary importance in South Africa.

PATHOGEN	MEDICAL / VETERINARY IMPORTANCE	REFERENCES
Banzi virus	Medical	Smithburn *et al.* 1959
Bunyamwera virus	Medical, veterinary (horses, cattle)	Kokernot *et al.* 1958, Tauro *et al.* 2015, Dutuze *et al.* 2020
Bwamba virus	Medical	Smithburn *et al.* 1941
Chikungunya virus	Medical	McIntosh *et al.* 1977, Schuffenecker *et al.* 2006
Dengue virus	Medical	Amarasinghe *et al.* 2011
Dirofilariasis	Medical, veterinary (dogs, cats)	Verster *et al.* 1991, Moodley *et al.* 2015
Germiston virus	Medical	Kokernot *et al.* 1960, Lambert & Lanciotti 2008
Middelburg virus	Veterinary (horses)	Attoui *et al.* 2007, Van Niekerk *et al.* 2015
Malaria	Medical	Gillies & De Meillon 1968
Pongola virus	Medical	Kalunda *et al.* 1985, Lambert & Lanciotti 2008
Rift Valley fever virus	Medical, veterinary (domestic ungulates)	Weiss *et al.* 1956, Jupp 1996
Semliki Forest virus	Medical	Mathiot *et al.* 1990
Sindbis virus	Medical, veterinary (horses)	Hubálek *et al.* 2014, Van Niekerk *et al.* 2015
Shuni virus	Veterinary (horses)	Van Eeden *et al.* 2012
Spondweni virus	Medical	McIntosh *et al.* 1961
Usutu virus	Medical	Engel *et al.* 2016
Wesselsbron virus	Medical, veterinary (domestic ungulates)	Weiss *et al.* 1956, Jupp 1996, Weyer *et al.* 2013
West Nile virus	Medical, veterinary (horses, dogs)	Ostlund *et al.* 2001, Lichtensteiger *et al.* 2003, Hart *et al.* 2014

1.1.2 ANTHROPOLOGICAL INFLUENCE ON MOSQUITO-BORNE DISEASES

It is crucial to consider the impact of anthropological activities on the prevalence, distribution and vector competence of mosquitoes. Economic and social progression may facilitate the transmission of mosquito-borne diseases, due to greater population densities, increased human mobility, international trade, habitat transformation and the accompanying ecological disruption (Braack *et al.* 2018). Other factors include the presence of sheltered habitats (Animut *et al.* 2013), the abundance of breeding sites (Calhoun *et al.* 2007) and urban heat islands, which may accelerate a vector's developmental rate (Shelton 1973, Oke 1973, Bayoh & Lindsay 2004). These elements provide ample opportunities for vector breeding and dispersal (Braack *et al.* 2018), which has become a common occurrence due to globalisation (Kampen *et al.* 2017).

The dispersal of vectors and their associated diseases can have devastating effects, since native organisms in newly infected regions are likely immunologically naive (Engel *et al.* 2016). Numerous authors documented the spread of mosquitoes to new geographic regions, including the introduction of *Cx.* (*Culex*) *quinquefasciatus* Say, 1823 to Australia, Jamaica and New Zealand (Belkin 1968a, Belkin *et al.* 1970), the introduction of *Ae.* (*Stegomyia*) *aegypti* (Linnaeus, 1762) to Jamaica and the New World (Belkin *et al.* 1970, Huang 1979) and the establishment of several non-native species in Germany (Kampen *et al.* 2017). Furthermore, *An.* (*Cellia*) *stephensi* was recently observed in northeastern Africa

for the first time, and as an urban-centred vector of malaria, it may hold prominent epidemiological implications for the region (Balkew *et al*. 2020, Sinka *et al*. 2020).

Climate change could also impact the dynamics of mosquito-borne diseases, since increases in ambient temperatures can influence the reproduction, development, growth rate and distribution of mosquitoes (Afrane *et al.* 2007, Afrane *et al*. 2012), while also potentially altering niche availability and the prevalence of predators (Bale *et al*. 2002). Since many localities across South Africa have experienced an increase in mean temperatures over the last few decades (Van Wilgen *et al*. 2016), climate change could impact mosquito vector development and allow these mosquitoes to populate previously uninhabitable regions (Githeko *et al*. 2000). This creates opportunities for range expansion, which could impact the epidemiology of mosquito-borne diseases and lead to new epidemics (Githeko *et al*. 2000).

1.2 MORPHOLOGY AND THE MOLECULAR APPROACH

1.2.1 SPECIES COMPLEXES AND MORPHOLOGICAL IDENTIFICATIONS

The successful implementation of vector control strategies relies on the knowledge of mosquito community compositions, species distributions, their contribution to local disease transmission dynamics and the accurate identification of vector species (Sallum *et al*. 2008, Weeraratne *et al*. 2018). However, vector species identifications are often complicated by morphologically similar or indistinguishable groups of species, which can still vary in their vectorial capacity (Gillies & De Meillon 1968, Koekemoer *et al*. 1999). Members within a species complex can differ in their distributions (Subbarao *et al*. 1988, Lühken *et al*. 2016), feeding habits (Joshi *et al*. 1988, Dusfour *et al.* 2004) and levels of insecticide resistance (Raghavendra *et al*. 1991, Dadzie *et al*. 2016), while sibling species can vary in their response to vector control efforts (Gillies & De Meillon 1968). Morphological identifications may require several life stages for accurate identifications and can be impeded by specimen damage and polymorphisms (Koekemoer *et al*. 1999, Lobo *et al*. 2015). Species boundaries between such morphologically similar groups may also be unclear. Therefore, Gillies and De Meillon (1968) emphasised the importance of recognising species as reproductively isolated units, as defined by the biological species concept (Mayr 2000).

1.2.2 APPLICATIONS OF MOLECULAR TECHNOLOGY

Since misidentifications of biological specimens are a relatively common occurrence, the ability to corroborate morphological identifications can serve as an invaluable tool to ensure the accuracy and validity of research efforts. Such morphological species identifications can be supplemented and substantiated with DNA barcoding, where DNA sequences are used to identify species (Hebert *et al.* 2003). DNA-based identifications can be a valuable addition to entomological surveillance programs (Zamora-Delgado *et al*. 2015), since it permits the identification of all life stages (Hebert *et al.* 2003). The molecular approach can also detect gene flow between groups (Hudson *et al.* 1992, Lee *et al.*

2013) and aid the recognition of species boundaries as a function of reproductive isolation (Gillies & De Meillon 1968, Mayr 2000, Alquezar *et al*. 2010). Since DNA sequences are often publicly accessible on DNA databases, the data can also be integrated into many other fields of research.

However, despite DNA barcoding's potential to provide an objective standard for identifications, the method nonetheless holds several limitations. These limitations include a large variability in pairwise distances, variation associated with specimen geography, limited database coverage, inaccurate data, non-universal similarity thresholds and the prominent overlap in dipteran intra- and interspecific COI variation (Meier *et al.* 2006). DNA barcoding also relies on the integrity of molecular databases, yet misidentified specimens are relatively common within such repositories (Bridge *et al*. 2003, Nilsson *et al*. 2006, Valkiūnas *et al.* 2008). Considering these limitations, barcoding results should be interpreted with caution and rather used to corroborate morphological identifications. However, despite the irregular success of COI in general dipteran identifications (Meier *et al.* 2006), DNA barcoding is still relatively effective in the identification of mosquito species (Meier *et al*. 2006, Versteirt *et al*. 2015).

The molecular approach remains a valuable asset within the field of taxonomy (Tautz *et al*. 2003, Blaxter 2004). The loss of type specimens, vague species descriptions and subjective interpretations are common challenges within the field of taxonomy (Edwards 1932). However, DNA can provide a relatively objective representation of a species and allow researchers to compare morphologically diverse and geographically distinct specimens (Ma & Xu 2005). Since many closely related species are morphologically indistinguishable, DNA analyses could also aid the resolution of species complexes (Saeung *et al.* 2008, Joyce *et al*. 2018).

DNA data can also be employed in molecular phylogenies to elucidate a taxon's evolutionary history. Phylogenetic results can give insight into the biodiversity, biogeography, adaptive radiation, cospeciation and evolutionary rates of organisms (Thorne *et al*. 1998, Huelsenbeck *et al*. 2000, Egan 2006). Phylogeny can also create a foundation for a clade's systematic structure (Harbach & Kitching 1998), which is especially relevant to Culicidae (mosquitoes) due to the family's volatile taxonomic and systematic history. Boundaries between closely related species are challenging to define, since differences may be life stage-specific or restricted to a particular sex (Edwards 1941, Koekemoer *et al.* 2002). The morphological diversity within higher taxonomic groups also complicates classifications and obscures taxonomic boundaries (Harbach 2007). Therefore, molecular phylogeny can provide insight into the evolutionary structure of mosquitoes and guide the refinement of its systematic framework, while the DNA barcoding can substantiate the identities of the specimens incorporated within the dataset.

1.2.3 PHYLOGENETIC UTILITY OF VARIOUS DNA REGIONS

Comprehensive phylogenetic analyses require a sufficient degree of taxonomic representation, and publicly available DNA sequences are commonly incorporated within such datasets. Since many genes are only represented by a relatively small number of sequenced specimens, it is worthwhile to select DNA regions that are well-represented within international databases, while ensuring that these regions are phylogenetically informative. The use of DNA regions with varying rates of evolution is also beneficial, since it ensures that the phylogenetic signal is available across a range of the taxon's evolutionary history. A few examples of applicable DNA regions include the mitochondrial cytochrome c oxidase subunit I (COI), nuclear second internal transcribed spacer (ITS2) and nuclear large subunit ribosomal RNA (28S) regions.

The COI region has been used in numerous culicid phylogenetic studies (Sallum *et al*. 2010, Weeraratne *et al*. 2018), since mitochondrial DNA provides phylogenetic resolution for lower taxonomic levels (Besansky & Fahey 1997). Here, the maternal inheritance of COI entails a linear evolutionary history, and the relatively high substitution rate can be used to categorise species (Beebe 2018), which ensures its utility as a DNA barcoding region. However, its ability to classify closely related species is limited, especially within mosquito species complexes (Beebe 2018). The second region, ITS2, has previously been used in polymerase chain reaction assays (PCR) to distinguish between members of *Anopheles* species groups and complexes (Li *et al.* 2005, Manonmani *et al.* 2007). Such ITS regions are under relatively low evolutionary constraints and diverge in a short period of time, therefore providing phylogenetic resolution for closely related species (Marinucci *et al*. 1999). Finally, the nuclear large ribosomal RNA gene (28S) has been used to investigate higher taxonomic relationships within Diptera, including the infraorder Culicomorpha (Pawlowski *et al*. 1996, Friedrich & Tautz 1997). Since this region is conserved in mosquitoes (Marinucci *et al*. 1999), it can provide phylogenetic resolution for relatively basal relationships.

1.2.4 MAXIMUM LIKELIHOOD AND BAYESIAN INFERENCE OF PHYLOGENY

Several molecular phylogenetic methods have been employed in the phylogenetic studies of Diptera (flies), including the maximum likelihood, maximum parsimony, neighbour-joining and Bayesian inferences of phylogeny (Friedrich & Tautz 1997, Marinucci *et al*. 1999, Garros *et al*. 2005, Zajac *et al*. 2016, Qilemoge *et al*. 2018). Authors frequently conduct Bayesian and maximum likelihood analyses, which both incorporate the observed data's probability, based on the generated tree (Felsenstein 1973, Huelsenbeck *et al*. 2002). The phylogenetic trees are often accompanied by measures of support, which reflect the tree's robustness against deviations (Egan 2006). Weak support values can also identify clades defined by conflicting and problematic characters (Kluge 1997).

Maximum likelihood trees often include bootstrap values as a measure of support. Bootstrapping generates pseudoreplicate data with randomly replaced characters, where both the original and

pseudoreplicate datasets are used to generate trees (DeSalle *et al*. 2002, Egan 2006). In effect, the duplication or removal of characters serves as a random weighting scheme (DeSalle *et al*. 2002). Multiple trees are generated from the data and the frequency with which a particular clade has been recovered is indicated by the bootstrap support value (DeSalle *et al*. 2002). On the other hand, Bayesian analyses make use of posterior probabilities as a measure of support. The posterior probabilities are investigated with a Marcov Chain Monte Carlo algorithm (Hastings 1970), where candidate trees are used to produce more accurate posterior probabilities (Yang & Rannala 1997). This process only investigates probable trees and is therefore very efficient (Yang & Rannala 1997), thus allowing large samples to be processed (Huelsenbeck *et al*. 2002). The parameter values are optimised during the initial burn-in period, after which the likelihood values stabilise and provide more accurate estimations of the posterior probabilities (Huelsenbeck *et al*. 2002). Both the Bayesian and maximum likelihood analyses generally produce similar topologies (Yang & Rannala 1997, Erixon *et al*. 2003), while nonparametric bootstrap values tend to be more conservative than posterior probabilities (Huelsenbeck *et al*. 2002).

1.2.5 RESEARCH GAPS WITHIN SOUTH AFRICAN MOSQUITO PHYLOGENETICS

Harbach (2007) emphasised the necessity to reassess culicid taxa to ensure their monophyly and establish boundaries between taxonomic groups. Mosquito systematics was traditionally based on superficial morphological similarity, which produced numerous non-monophyletic taxa, as demonstrated by modern analyses (Harbach 2007). Fortunately, taxonomy was revolutionised by the integration of molecular approaches, which led to the discovery of species complexes and provided more objective measures for species delineation (Harbach 2007).

Culicidae's phylogenetic examinations were historically focused on species of medical and veterinary importance, while non-vector species received much less attention (Besansky & Fahey 1997). Additionally, molecular phylogenetic studies involving *Anopheles, Aedes* and *Culex* often examined the higher-level relationships between genera and subfamilies, and included minimal representation within each genus (Besansky & Fahey 1997, Shepard *et al.* 2006). This likely impacted the phylogenetic results, since analyses require sufficient representation within each clade for a robust confirmation of its monophyly (Miller *et al*. 1997).

Researchers also generally focused on *Anopheles* mosquitoes due to their role as vectors of malaria (Harbach 2007). However, to date, no phylogenetic studies have focused on South African *Aedes* and *Culex* species, while only a handful of studies examined the phylogeny of South African *Anopheles* (Koekemoer *et al*. 2009, Norris & Norris 2015). The lack of molecular research on South African culicids is exemplified by the relative shortage of records on repositories such as Barcode Of Life Data System v4 (BOLD Systems 2020) and GenBank (Sayers *et al*. 2019). Many of these South African sequences belong to species of medical and veterinary importance, while non-vectors are generally

underrepresented. Therefore, relatively little is known about the molecular diversity, affiliations and evolutionary history of South African mosquito species.

1.3 STUDY AIMS AND OBJECTIVES

The current study investigated the intrageneric relationships of South African *Anopheles, Aedes* and *Culex* mosquitoes to shed light on their affiliations and broader placement within the culicid systematics.

- To achieve this aim, mosquitoes were sampled in central South Africa across diverse habitats, including rural, agricultural and urban localities, to supplement the relatively poor coverage of South African mosquitoes within the DNA repositories. Additionally, the sampling served as an opportunity to contribute up-to-date distribution records of the sampled species in the region.

- In order to examine the full diversity of sampled specimens, numerous morphotypes from various localities were sequenced for the three target DNA regions (COI, ITS2 and 28S). The sequences were subsequently used to verify the identity of the specimens.

- DNA sequences of the target regions were obtained from international DNA databases (GenBank and BOLD) to represent the major culicid taxonomic divisions, in addition to all available South African species of *Anopheles*, *Aedes* and *Culex*.

- Bayesian and maximum likelihood phylogenetic analyses were conducted for each genus and gene region, based on datasets consisting of the generated and database DNA sequences. The results were examined for common and well-supported relationships, which represented a common phylogenetic signal shared between the methods and DNA regions.

1.4 CULICIDAE SYSTEMATICS AND TAXONOMY

The systematic literature provided insight into the history and progression of Culicidae's systematic framework. Its history represents shifting viewpoints as new taxonomic approaches were incorporated, exemplifying the historical and current ambiguity associated with many of Culicidae's subgeneric classifications. The family has a relatively extensive taxonomic history, involving ongoing debates on the boundaries and placement of multiple taxa within the family, in addition to the family itself. Dyar and Knab (1906) already had a relatively contemporary view on Culicidae, where they considered Chaoboridae (Corethridae) and Dixidae separate from mosquitoes based on larval characteristics. The authors also divided Culicidae into the three subfamilies, namely Anophelinae, Culicinae and Sabethinae. Theobald (1907) supported the separation of chaoborids from Culicidae and recognised the subfamilies of Dyar and Knab (1906). However, not all authors agreed with this definition of culicids. Ross (1951) included Chaoboridae as a subfamily of Culicidae, while Edwards (1932) and Belkin

(1968a) included both choaborids and dixids as subfamilies of Culicidae. Nonetheless, subsequent authors acknowledged the separation of Culicidae from these closely related families. This included Tanaka *et al*. (1979), who adopted the modern concept of Culicidae and recognised two subfamilies, Anophelinae and Culicinae.

Culicidae currently consists of about 3,490 species, which likely underestimates the true diversity of mosquitoes, due to the presence of species complexes (Harbach 2007). Morphological and molecular data support the monophyly of the family (Miller *et al*. 1997, Harbach & Kitching 1998) in addition to certain subfamilial clades, such as Culicini and Anophelinae (Harbach & Kitching 1998, Krzywinski *et al*. 2001). Miller *et al*. (1997) also considered *Aedes* and *Culex* monophyletic assemblages, while Harbach and Kitching (1998) noted that female and pupal morphology could support *Culex*'s polyphyly. Harbach and Kitching (1998) also noted that polymorphisms of the tribe Aedini, including *Aedes*, obscure their intergeneric relationships. Since clades within the tribe Aedini overlap morphologically, it necessitates the use of character combinations to define groupings (Harbach 2007). This degree of overlap has led to considerable ambiguity in the classification of both *Aedes* and *Culex* (Harbach 2007).

The uncertainty of the family's subfamilial boundaries has persisted throughout the last century, which demonstrates the subject's complexity. The comprehensive taxonomic investigations of Culicidae were instigated in the early 1900s, when Theobald described numerous mosquito species. During the emergence of the field, Theobald (1907) recognised the implications of colour variants on species delineations, highlighting one of the many challenges associated with morphology-based taxonomy. Theobald's (1910) classification included numerous subdivisions within the family, with a total of ten subfamilies and more than 140 genera. Edwards (1932) subsequently condensed the family into 30 genera, in an attempt to facilitate the delineations of taxa and represent their broader relationships. Here, many of Theobald's (1910) genera were incorporated as subdivisions of genera within Edward's (1932) classification. Edward's (1932) generic classifications were based on characteristics shared between the sexes and were more closely aligned with the modern classification of 41 genera.

1.4.1 *AEDES*

The taxonomic history of *Aedes* included prominent disputes on taxon borders, which is exemplified by the use of two different classification schemes for the tribe Aedini. These include the proposals of Reinert *et al*. (2004, 2006, 2008, 2009) involving numerous modifications to the structure of the tribe and the conservative proposal of Wilkerson *et al*. (2015), which maintains the traditional structure of Aedini. The proposals of Reinert *et al*. (2004, 2006, 2008, 2009) elevated many *Aedes* subgenera and portions thereof to generic status. This radical adjustment to Aedini's classification left taxonomists divided on its validity, which held subsequent implications for the taxa involved. Publications subsequently employed different generic names for the same species, including *Aedes* / *Stegomyia aegypti*, *Aedes* / *Aedimorphus vexans* (Meigen, 1830) and *Aedes* / *Ochlerotatus triseriatus* (Say, 1823) (Bara & Muturi 2015, Garigliany *et al*. 2015, Kraemer *et al*. 2015, Westby *et al*. 2015, Naeem *et al*.

2016, Schönonberger *et al.* 2016). This lack of consensus likely had ramifications for the dissemination of information on medically relevant species (Savage & Strickman 2004).

According to Wilkerson *et al.* (2015), their reanalyses of Reinert *et al.*'s (2009) data yielded weak support for its phylogenetic results. Wilkerson *et al.* (2015) believed that the new classification was impractical and hindered effective communication, and therefore restored *Aedes* to its traditional status. To guarantee the stability of systematic adjustments, a robust and relatively undisputed framework is required before major changes are implemented. Therefore, the current study follows the system proposed by Wilkerson *et al.* (2015) and Wilkerson and Linton (2015), which mainly retains the traditional structure of *Aedes*. This classification also includes the two recently described taxa, *Pseudalbuginosus* Huang & Rueda, 2015 and *Paulianius* Brunhes & Boussès, 2017 (Harbach 2020a), which are considered as subgenera of *Aedes*. In total, this classification includes 78 subgenera within *Aedes* (Fig. 1.1).

FAMILY	Culicidae
SUBFAMILY	Culicinae
TRIBE	Aedini
GENUS	*Aedes*
SUBGENUS (A-D)	*Abraedes*, *Acartomyia*, *Aedes*, *Aedimorphus*, *Alanstonea*, *Albuginosus*, *Ayurakitia*, *Aztecaedes*, *Belkinius*, *Bifidistylus*, *Borichinda*, *Bothaella*, *Bruceharrisonius*, *Cancraedes*, *Catageiomyia*, *Catatassomyia*, *Christophersiomyia*, *Coetzeemyia*, *Collessius*, *Cornetius*, *Dahliana*, *Danielsia*, *Dendroskusea*, *Diceromyia*, *Dobrotworskyius*, *Downsiomyia*
FAMILY	Culicidae
SUBFAMILY	Culicinae
TRIBE	Aedini
GENUS	*Aedes*
SUBGENUS (E-MA)	*Edwardsaedes*, *Elpeytonius*, *Finlaya*, *Fredwardsius*, *Georgecraigius*, *Geoskusea*, *Gilesius*, *Gymnometopa*, *Halaedes*, *Himalaius*, *Hopkinsius*, *Howardina*, *Huaedes*, *Hulecoeteomyia*, *Indusius*, *Isoaedes*, *Jarnellius*, *Jihlienius*, *Kenknightia*, *Kompia*, *Leptosomatomyia*, *Levua*, *Lewnielsenius*, *Lorrainea*, *Luius*, *Macleaya*
FAMILY	Culicidae
SUBFAMILY	Culicinae
TRIBE	Aedini
GENUS	*Aedes*
SUBGENUS (MO-Z)	*Molpemyia*, *Mucidus*, *Neomelaniconion*, *Nyctomyia*, *Ochlerotatus*, *Paraedes*, *Patmarksia*, *Paulianius*, *Petermattinglyius*, *Phagomyia*, *Polyleptiomyia*, *Protomacleaya*, *Pseudalbuginosus*, *Pseudarmigeres*, *Pseudoskusea*, *Rampamyia*, *Rhinoskusea*, *Rusticoidus*, *Sallumia*, *Scutomyia*, *Skusea*, *Stegomyia*, *Tanakaius*, *Tewarius*, *Vansomerenis*, *Zavortinkius*

Fig. 1.1. Major taxonomic divisions of the genus *Aedes* according to the updated traditional classification, adapted from Harbach (2020a).

1.4.1.1 *AEDES* TAXONOMIC OVERVIEW

Aedes was initially described as a genus by Meigen (1818), which was followed by the description of numerous relevant genera, including *Ochlerotatus* Lynch Arribálzaga, 1891, *Gymnometopa* Coquillett, 1906, *Neomelaniconion* Newstead, 1907, *Christophersiomyia* Barraud, 1923, *Catatassomyia* Dyar and Shannon, 1925, *Paraedes* Edwards, 1934 , *Ayurakitia* Thurman, 1954 and a multitude of genera described by Theobald (1901a, 1903a, 1903b, 1903c, 1904, 1905a, 1905b, 1907, 1910, 1911), including *Acartomyia, Aedimorphus, Catageiomyia*, *Danielsia, Diceromyia, Finlaya, Howardina, Hulecoeteomyia, Leptosomatomyia, Macleaya, Molpemyia, Mucidus, Phagomyia, Polyleptiomyia, Protomacleaya, Pseudoskusea, Skusea, Scutomyia* and *Stegomyia*. These genera were subsequently considered as subgenera of *Aedes*.

Authors also gained a greater interest in the description of novel *Aedes* subgenera, including Edwards (1929, 1930), who described numerous subgenera (*Cancraedes, Dendroskusea, Dunnius, Geoskusea* and *Rhinoskusea*). Stone and Knight (1956) noted that the name *Dunnius* was already in use and proposed the replacement name *Pseudarmigeres*. Several other authors also contributed descriptions of subgenera, including *Indusius* Edwards, 1934, *Kompia* Aitken, 1941, *Levua* Stone and Bohart, 1944, *Downsiomyia* Vargas, 1950, *Alanstonea* Mattingly, 1960, *Huaedes* Huang, 1968, *Abraedes* Zavortink, 1970, *Aztecaedes* Zavortink, 1972, *Rusticoidus* Shevchenko and Prudkina, 1973 and three subgenera described by Belkin (1962), including *Edwardsaedes*, *Halaedes* and *Lorrainea*.

Since the 1970s, the taxonomic work on Aedini was predominantly conducted by Reinert (1973a, 1979, 1982, 1987, 1990, 1999, 2000a), who described multiple *Aedes* subgenera, including *Albuginosus, Belkinius, Bothaella, Fredwardsius, Isoaedes, Kenknightia* and *Zavortinkius*. Reinert (2000b) also elevated the subgenus *Ayurakitia* to generic rank, divided *Aedes* into two separate genera, *Aedes* and *Ochlerotatus* (2000c), and described *Bruceharrisonius* as a subgenus of *Ochlerotatus* (2003). However, Savage and Strickman (2004) did not agree with the bisection of *Aedes* and believed that such taxonomic adjustments required a more robust classification. The authors also believed that *Aedes* and *Ochlerotatus* possessed enough unifying characteristics to justify *Ochlerotatus*' status as a subgenus of *Aedes*.

Nevertheless, Reinert *et al*. (2004) proposed additional changes to the tribe. The tribe initially consisted of 12 genera, while Reinert *et al*.'s (2004) proposal elevated 32 *Aedes* and *Ochlerotatus* subgenera to generic rank (*Abraedes, Aedes, Alanstonea, Albuginosus, Aztecaedes, Belkinius, Bothaella, Christophersiomyia, Diceromyia, Edwardsaedes, Fredwardsius, Geoskusea, Gymnometopa, Halaedes, Howardina, Huaedes, Indusius, Isoaedes, Kenknightia, Kompia, Leptosomatomyia, Levua, Lorrainea, Mucidus, Neomelaniconion, Paraedes, Pseudarmigeres, Rhinoskusea, Scutomyia, Skusea, Stegomyia* and *Zavortinkius*). Additionally, three *Ochlerotatus* clades received generic status (*Finlaya, Downsiomyia* and the novel genus *Tanakaius*) and one *Ochlerotatus* subgenus was included within the genus *Opifex* Hutton, 1902. This was followed by the description of two *Aedes* subgenera, *Cornetius*

Huang, 2005 and *Coetzeemyia* Huang, Mathis and Wilkerson, 2010, in addition to the description of three genera, *Tewarius* Reinert, 2006, *Borichinda* Harbach and Rattanarithikul, 2007 and *Nyx* Harbach and Linton, 2013. Since the name *Nyx* was already occupied, *Nyctomyia* was suggested as a replacement name (Harbach 2013).

Reinert *et al*. (2006, 2008, 2009) continued to restructure Aedini in three additional papers. Reinert *et al*. (2006) elevated *Bruceharrisonius, Macleaya, Molpemyia* and *Pseudoskusea* to generic rank, restored the generic status of *Hulecoeteomyia* and *Phagomyia*, established 11 novel genera (*Collessius, Dahliana, Dobrotworskyius, Georgecraigius, Gilesius, Himalaius, Jarnellius, Jihlienius, Patmarksia, Rampamyia* and *Vansomerenis*) and described *Lewnielsenius* as a subgenus of *Jarnellius*. Reinert *et al*. (2008) included *Acartomyia* and the novel subgenus *Sallumia* as subgenera of *Ochlerotatus*, restored *Danielsia* to generic rank and established two new genera (*Luius* and *Hopkinsius*). Finally, Reinert *et al*. (2009) elevated nine subgenera to generic rank (*Acartomyia, Aedimorphus, Cancraedes, Cornetius, Geoskusea, Levua, Lewnielsenius, Rhinoskusea* and *Sallumia*), described three novel genera (*Bifidistylus, Elpeytonius* and *Petermattinglyius*), resurrected *Catageiomyia, Catatassomyia, Diceromyia* and *Polyleptiomyia* from synonymy and established these taxa as genera.

However, Wilkerson *et al*. (2015) did not agree with the structure proposed by Reinert *et al*. (2004, 2006, 2008, 2009). The authors restored the classification of Aedini to its standing prior to the year 2000 and considered the novel aedine genera as subgenera of *Aedes*. This was followed by Wilkerson and Linton's (2015) reinstatement of *Pseudoskusea, Protomacleaya* and *Rusticoidus* as subgenera of *Aedes*. Two aedine taxa have subsequently been described, including the monotypic subgenus *Pseudalbuginosus* Huang and Rueda, 2015 and the genus *Paulianius* Brunhes and Boussès, 2017. Therefore, both *Pseudalbuginosus* and *Paulianius* are considered to be subgenera of *Aedes* according to Wilkerson *et al*.'s (2015) classification.

1.4.1.2 *AEDES* SUBGENERA RELEVANT TO SOUTH AFRICA

Jupp (1996) originally listed ten *Aedes* subgenera occurring in South Africa (*Aedimorphus, Albuginosus, Diceromyia, Finlaya, Levua, Mucidus, Neomelaniconion, Ochlerotatus, Pseudarmigeres* and *Stegomyia*). This was followed by the description of several relevant taxa, including the descriptions of *Bifidistylus* Reinert, Harbach and Kitching, 2009 and *Hopkinsius* Reinert, Harbach and Kitching, 2008 as genera, and *Fredwardsius* Reinert, 2000 and *Zavortinkius* Reinert, 1999 as subgenera. Eventually, all South African representatives of *Finlaya* were transferred to *Hopkinsius* and *Zavortinkius*, while several *Aedimorphus* species were included within *Catageiomyia* and *Polyleptiomyia*. Huang *et al*. (2010) also transferred *Ae. fryeri* (Theobald, 1912) from *Levua* to the novel subgenus *Coetzeemyia*, thus withdrawing the only South African representative of the subgenus.

Subgenus *Aedimorphus*

Aedimorphus was initially described as a genus by Theobald (1903a) and was considered a subgenus of *Aedes* by Edwards (1932), who divided it into eight groups (Group A–H). Knight and Hurlbut (1949) believed that Edward's Group C and D were sufficiently similar to justify its treatment as a single group. McIntosh (1975) then divided southern African *Aedimorphus* species into seven groups, which resembled five of Edward's divisions. Reinert (2000d) also divided *Aedimorphus* into three species assemblages (Domesticus, Alboscutellatus and Argenteopunctatus) and divided Domesticus into subassemblages (Domesticus, Vexans and Mediolineatus). This was followed by Reinert *et al.*'s (2009) elevation of *Aedimorphus* to generic rank, which was subsequently reverted to its current subgeneric status by Wilkerson *et al.* (2015).

Subgenus *Albuginosus*

Albuginosus was described as a subgenus of *Aedes* by Reinert (1987) and consisted of species previously assigned to *Aedimorphus*. Reinert *et al.* (2004) later elevated *Albuginosus* to generic rank, while Wilkerson *et al.* (2015) once again reverted it to subgeneric status.

Subgenus *Bifidistylus*

Reinert *et al.* (2009) initially described the genus *Bifidistylus* for a type species previously included in *Aedimorphus*. However, after Wilkerson *et al.*'s (2015) reanalysis of Reinert *et al.*'s (2009) data, the genus was reduced to a subgenus of *Aedes*.

Subgenus *Catageiomyia*

Theobald (1903c) described *Catageiomyia* as a genus, which was subsequently synonymised with *Aedimorphus*. Reinert *et al.* (2009) removed *Catageiomyia* from synonymy and reinstated it as a genus, while Wilkerson *et al.* (2015) once again considered it a subgenus of *Aedes*.

Subgenus *Coetzeemyia*

Levua was described as a subgenus of *Aedes* by Stone and Bohart (1944) and after *Ochlerotatus*' elevation to generic rank, it was included as a subgenus of *Ochlerotatus*' Section I (Reinert 2000c). Huang *et al.* (2010) subsequently described *Coetzeemyia* as a subgenus of *Aedes* and transferred the only South African representative of *Levua* to the novel monotypic subgenus. Finally, *Coetzeemyia*'s subgeneric status was retained in Wilkerson *et al.*'s (2015) classification.

Subgenus *Diceromyia*

Diceromyia was originally described as a genus by Theobald (1911) and was subsequently considered a subgenus of *Aedes* by Edwards (1932), who divided it into two groups (Diceromyia and Dendroskusea). Reinert *et al*. (2004) later restored *Diceromyia* to its generic rank, while Wilkerson *et al*. (2015) reinstated it as a subgenus of *Aedes*.

Subgenus *Fredwardsius*

Reinert (2000a) erected *Fredwardsius* as a monotypic subgenus of *Aedes,* which was subsequently elevated to generic rank by Reinert *et al*. (2004). However, Wilkerson *et al*. (2015) once again reduced *Fredwardsius* to its subgeneric status.

Subgenus *Hopkinsius*

Hopkinsius was recently established as a genus by Reinert *et al*. (2008) for a clade consisting of *Finlaya* species. Wilkerson *et al.* (2015) subsequently reduced the taxon to its subgeneric status within *Aedes,* while Huang and Rueda (2017) considered *Hopkinsius* as a part of *Finlaya*, based on their male genital characteristics.

Subgenus Mucidus

Mucidus and *Pardomyia* were originally described as genera by Theobald (1901a, 1907), while Edwards (1932) considered *Mucidus* as a subgenus of *Aedes* and divided it into two groups (Mucidus and Pardomyia). *Mucidus* was subsequently included as a subgenus of the newly elevated *Ochlerotatus* (Reinert 2000c). Reinert *et al.* (2004) then restored *Mucidus* to generic rank, while Wilkerson *et al*. (2015) reverted it to subgeneric status.

Subgenus *Neomelaniconion*

Newstead *et al*. (1907) listed a novel species within the genus *Neomelaniconion*, where it was denoted with an asterisk, relating the genus to Theobald. During the same year, *Banksinella* was described as a genus by Theobald (1907). *Banksinella* was subsequently considered a synonym of *Neomelaniconion*, and Newstead was credited for the taxon. Edwards (1941) still referred to the name *Banksinella* and considered it a subgenus of *Aedes*, while subsequent authors acknowledged *Neomelaniconion* as the subgeneric name. Reinert *et al*. (2004) later elevated *Neomelaniconion* to generic rank, while Wilkerson *et al.* (2015) reinstated its subgeneric status.

Subgenus *Ochlerotatus*

Ochlerotatus was originally described as a genus by Lynch Arribálzaga (1891). Edwards (1932) subsequently considered *Ochlerotatus* as a subgenus of *Aedes* and divided it into eight groups, while Zavortink (1972) included two sections within the subgenus (Pulchritarsis and Atropalpus). Reinert (2000c) elevated *Ochlerotatus* to generic rank, where it was divided into two sections based on the genitalia and immature characteristics. *Ochlerotatus* Section I consisted of 16 subgenera, including *Finlaya, Geoskusea, Halaedes, Kenknightia, Levua, Macleaya, Molpemyia, Mucidus, Ochlerotatus, Protomacleaya, Pseudoskusea, Rhinoskusea, Rusticoidus* and *Zavortinkius*, while *Ochlerotatus* Section II included *Abraedes, Aztecaedes, Gymnometopa, Howardina* and *Kompia* as subgenera.

Reinert *et al*. (2004) subsequently elevated several *Ochlerotatus* subgenera to generic rank (*Abraedes, Aztecaedes, Gymnometopa, Kompia, Levua, Geoskusea, Halaedes, Howardina, Kenknightia, Mucidus, Rhinoskusea* and *Zavortinkius*). The authors also assigned generic rank to three additional *Ochlerotatus* clades (*Finlaya, Downsiomyia* and the novel genus *Tanakaius*) and divided the subgenus *Ochlerotatus* into three lineages. The generic status of *Ochlerotatus* was not widely acknowledged, since many authors were convinced that the genital features did not provide sufficient support for this adjustment (Harbach 2007). Therefore, many subsequent publications still treated *Ochlerotatus* as a subgenus of *Aedes* (Lozano-Fuentes *et al*. 2012, Huang & Rueda 2014, Jardine *et al*. 2014) and Wilkerson *et al*. (2015) formally reinstated *Ochlerotatus*' subgeneric rank.

Subgenus *Polyleptiomyia*

Polyleptiomyia was described as a genus by Theobald (1905b) and was subsequently synonymised with *Aedimorphus* (Reinert 1973b). *Polyleptiomyia* was later reinstated as a genus by Reinert *et al.* (2009) and finally reduced to subgeneric rank by Wilkerson *et al*. (2015).

Subgenus *Pseudarmigeres*

Edwards (1930) initially described *Dunnius* as a subgenus of *Aedes*, where the taxon name was already in use. Therefore, Stone and Knight (1956) proposed *Pseudarmigeres* as a replacement name. Reinert *et al.* (2004) later elevated the subgenus to generic rank, while Wilkerson *et al.* (2015) reinstated it as a subgenus of *Aedes*.

Subgenus *Stegomyia*

Theobald (1901a) originally described *Stegomyia* as a genus, while Edwards (1932) subsequently considered it as a subgenus of *Aedes* and divided *Stegomyia* into four groups (Aegypti, W-alba, Scutellaris and *Aedes vittatus*). Huang (1988, 1990, 1997) later established three additional groups (Pseudonigeria, Africanus and Dendrophilus) and subsequently (Huang 2004) recognised 11

Afrotropical *Stegomyia* groups (Aegypti, Africanus, Apicoargenteus, Dendrophilus, Granti, Metallicus, Poweri, Pseudonigeria, Scutellaris, Simpsoni and Unilineatus). Reinert *et al*. (2004) then restored *Stegomyia* to generic rank, while Wilkerson *et al*. (2015) reinstated its subgeneric status.

Subgenus *Zavortinkius*

Reinert (1999) initially described *Zavortinkius* as a subgenus of *Aedes*, and later considered it a subgenus of *Ochlerotatus* (Reinert 2002). Reinert *et al*. (2004) subsequently elevated *Zavortinkius* to generic rank, which was once again reduced to subgeneric status by Wilkerson *et al*. (2015).

1.4.2 *CULEX*

Culex, as the type genus of Culicidae, has a relatively extensive taxonomic history. Despite its description in 1758, most taxonomic efforts were pursued in the 1900s. These taxonomic efforts consequently shaped the current classification of *Culex*, consisting of 26 subgenera (Fig. 1.2).

FAMILY	Culicidae
SUBFAMILY	Culicinae
TRIBE	Culicini
GENUS	*Culex*
SUBGENUS	*Acalleomyia*, *Acallyntrum*, *Aedinus*, *Afroculex*, *Allimanta*, *Anoedioporpa*, *Barraudius*, *Belkinomyia*, *Carrollia*, *Culex*, *Culiciomyia*, *Eumelanomyia*, *Kitzmilleria*, *Lasiosiphon*, *Lophoceraomyia*, *Maillotia*, *Melanoconion*, *Micraedes*, *Microculex*, *Neoculex*, *Nicaromyia*, *Oculeomyia*, *Phenacomyia*, *Phytotelmatomyia*, *Sirivanakarnius*, *Tinolestes*
SECTION	Melanoconion, Spissipes (subgenus *Melanoconion*)
SERIES	Consolator, Imitator, Inimitabilis, Pleuristriatus (subgenus *Microculex*)

Fig. 1.2. Major taxonomic divisions of the genus *Culex*, adapted from Harbach (2020b).

1.4.2.1 *CULEX* TAXONOMIC OVERVIEW

Culex was established by Linnaeus (1758) during the emergence of taxonomy, which entailed relatively rudimentary descriptions. The initial portrayal of *Culex* was broad and applicable to many mosquito taxa (Edwards 1932), yet it fashioned a foundation for the subsequent taxonomic efforts in the early 1900s. Numerous relevant taxa were initially described as genera, including *Melanoconion* Theobald, 1903, *Aedinus* Lutz, 1904, *Carrollia* Lutz, 1905, *Lophoceraomyia* Theobald, 1905, *Neoculex* Dyar, 1905, *Micraedes* Coquillett, 1906, *Tinolestes* Coquillett, 1906, *Culiciomyia* Theobald, 1907, *Maillotia*

Theobald, 1907, *Microculex* Theobald, 1907, *Oculeomyia* Theobald, 1907, *Acalleomyia* Leicester, 1908 and *Eumelanomyia* Theobald, 1909.

Many taxa were also subsequently considered subgenera of *Culex*, including *Aedinus*, *Culiciomyia*, *Lophoceraomyia* (*Lophoceratomyia*), *Melanoconion, Micraedes, Microculex, Neoculex* and *Tinolestes* (Edwards 1917, 1921, 1930; Dyar 1918), while Edwards (1922) alluded to *Eumelanomyia*'s potential as a subgenus. Authors also developed a greater interest in the description of novel *Culex* subgenera, resulting in the descriptions of *Barraudius* Edwards, 1921, *Anoedioporpa* Dyar, 1923, *Lasiosiphon* Kirkpatrick, 1925 and *Acallyntrum* Stone and Penn, 1948. Dyar (1928) also listed several sections within the subgenus *Melanoconion*, including Tinolestes, Melanoconion and Anoedioporpa.

Edwards (1932) similarly characterised 16 *Culex* subgenera, based primarily on the male genitalia (*Acalleomyia, Aedinus, Barraudius, Carrollia, Culex, Culiciomyia*, *Lasiosiphon, Lophoceraomyia* [*Lophoceratomyia*], *Melanoconion, Micraedes, Microculex* and *Neoculex*) and included *Eumelanomyia* as a group within *Neoculex*. Belkin (1968b) later subdivided Neotropical *Aedinus* species into several subgenera (*Anoedioporpa, Melanoconion*, *Micraedes* and *Tinolestes*). Sirivanakarn (1971, 1976) ultimately recognised *Eumelanomyia* and *Maillotia* as subgenera and synonymised *Oculeomyia* with the subgenus *Culex*. Tanaka (2004) subsequently resurrected *Oculeomyia* from synonymy.

The latter half of the century and early 2000s similarly yielded numerous subgeneric descriptions, including *Allimanta* Casal and García, 1968, *Belkinomyia* Adames and Galindo, 1973, *Afroculex* Danilov, 1989, *Kitzmilleria* Danilov, 1989, *Phenacomyia* Harbach and Peyton, 1992, *Nicaromyia* González Broche and Rodríguez Rodríguez, 2001, *Sirivanakarnius* Tanaka, 2004 and *Phytotelmatomyia* Rossi and Harbach, 2008, thus resulting in the current classification of 26 subgenera.

1.4.2.2 *CULEX* SUBGENERA RELEVANT TO SOUTH AFRICA

Jupp (1996) originally listed six *Culex* subgenera occurring in southern Africa, including *Afroculex, Culex, Culiciomyia, Eumelanomyia* and *Maillotia*. Jupp (1996) also listed *Lutzia* Theobald, 1903 as a subgenus of *Culex*, however *Lutzia* was subsequently elevated to generic rank (Tanaka 2003). Additionally, a single southern African species was later transferred to the subgenus *Oculeomyia* (Tanaka 2004).

Subgenus *Afroculex*

The subgenus *Afroculex* was described by Danilov (1989), where the type was transferred between several other taxa prior to the subgenus' description. Here, *Cx. lineatus* (Theobald, 1912) was moved from *Pseudohowardina* to *Culex* (Edwards 1914), then provisionally to *Neoculex* (Edwards 1941) and later to *Maillotia* (Sirivanakarn 1971). The type species was finally transferred to *Afroculex* in 1989.

Subgenus *Culex*

Edwards (1932) bisected the subgenus *Culex* into two groups, Sitiens and Pipiens, with each group consisting of four series. Three additional groups were later recognised, including the Guiarti, Atriceps and Duttoni Groups (Edwards 1941, Belkin 1962, Harbach 1988). Sirivanakarn's (1976) subdivisions differed from Edward's and consisted of subgroups instead of series, where the Pipiens and Sitiens Groups contained four and six subgroups, respectively. As a result, the current classification consists of the Atriceps, Duttoni, Guiarti, Pipiens and Sitiens Groups.

Subgenus *Culiciomyia*

Culiciomyia was initially described as a genus by Theobald (1907) and was subsequently considered a subgenus by Edwards (1921). Edwards (1932) divided the subgenus into two groups (Fragilis and Nebulosus), while subsequent authors also recognised the Dispectus (Bram 1969) and Tricuspis (Harrison 1987) Groups. Taxonomists additionally acknowledged a group of closely related species within *Culiciomyia* (Sirivanakarn 1977), which was later named the Shebbearei Group (Harbach 2011). Therefore, *Culiciomyia* currently consists of the Fragilis, Dispectus, Nebulosus, Shebbearei and Tricuspis Groups.

Subgenus *Eumelanomyia*

Eumelanomyia and *Protomelanoconion* were described as a genera by Theobald (1909, 1910) and noted as potential subgenera of *Culex* by Edwards (1922). Edwards (1930) also described *Mochthogenes* as a subgenus of *Culex*. Sirivanakarn (1971) later listed four groups within *Eumelanomyia* (Eumelanomyia, Mochthogenes, Protomelanoconion and Rubinotus-rima) with Rubinotus-rima and Mochthogenes subdivided into two and eight subgroups, respectively. Sirivanakarn (1972) eventually adjusted Mochthogenes' classification, resulting in a total of 13 subgroups. The groups and subgroups of Sirivanakarn were subsequently retained in the current classification.

Subgenus *Maillotia*

Maillotia was described as a genus by Theobald (1907), where the type species was subsequently transferred to *Culex* (Edwards 1911). Sirivanakarn (1971) considered *Maillotia* as a subgenus of *Culex* and listed several groups within the subgenus. This included the Hortensis and Seyrigi Groups, which were retained in the current classification.

Subgenus *Oculeomyia*

Oculeomyia was originally established as a genus by Theobald (1907), where the type species was successively synonymised with *Cx. ager* Giles, 1901 (Edwards 1911) and *Cx. bitaeniorhynchus* Giles, 1901 (Edwards 1913), thus synonymising *Oculeomyia* with *Culex*. Edwards (1932) later assigned *Cx.*

bitaeniorhynchus to the Bitaeniorhynchus Series within the Sitiens Group. The type species was subsequently transferred to *Oculeomyia,* therefore resurrecting it from synonymy and establishing it as a subgenus (Tanaka 2004).

1.4.3 *ANOPHELES*

Anopheles was described as a genus by Meigen (1818) and consists of eight subgenera (Fig. 1.3). The use of *Anopheles*' taxonomic rankings has shifted throughout its taxonomic history, where groups were initially used as the primary divisions of subgenera. However, Reid and Knight (1961) preferred sections over groups, which in turn were subdivided into series. Gillies and De Meillon (1968) similarly favoured other divisions over groups, but used sections as subdivisions of series, in contrast with Reid and Knight (1961). Nonetheless, the current classification aligns with the structure of Reid and Knight (1961), where subgenera are divided into sections, and sections into series.

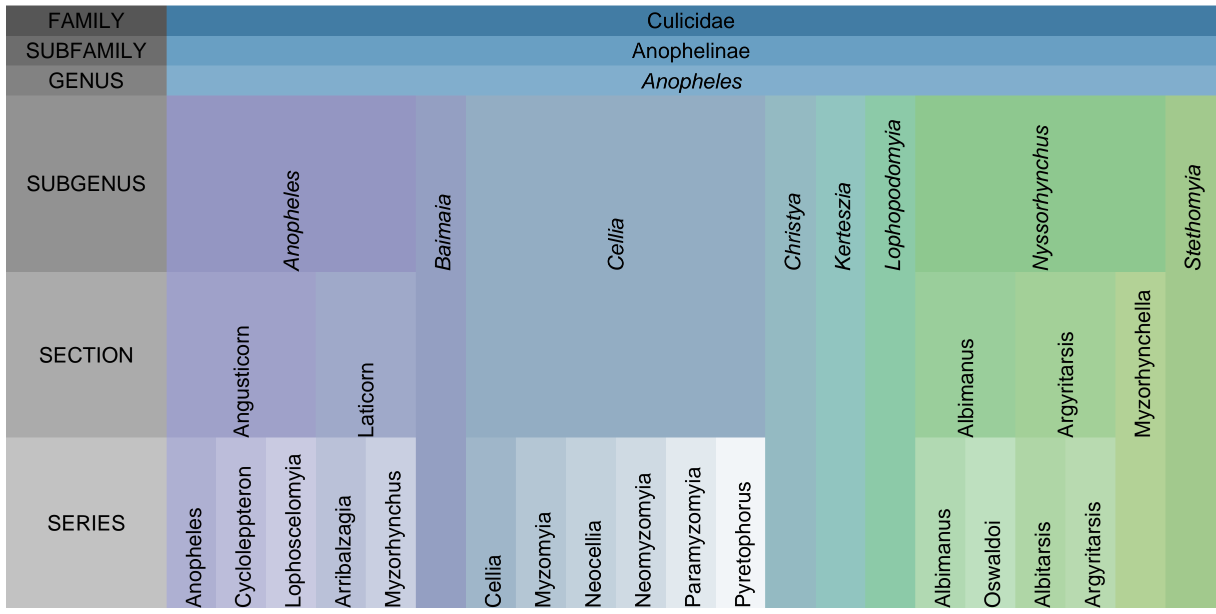

Fig. 1.3. Major taxonomic divisions of the genus *Anopheles*, adapted from Harbach (2020c).

Subgenus *Anopheles*

The description of *Anopheles* was followed by the descriptions of several other genera by Theobald (1901b, 1902, 1903d, 1904), including *Arribalzagia*, *Christya*, *Cycloleppteron*, *Lophoscelomyia* and *Rossia,* in addition to the designation of *Myzorhynchus* as a replacement name for *Rossia* (Blanchard 1902). This was followed by the subdivision of the subgenus *Anopheles*, where Root (1923) and Christophers (1924) established three groups (Anopheles, Arribalzagia and Christya). Edwards (1932) similarly recognised Anopheles, Arribalzagia and Christya as groups and listed four series within the Anopheles Group (Anopheles, Myzorhynchus, Lophoscelomyia and Cycloleppteron).

Reid and Knight (1961) divided the subgenus into two sections. The Laticorn Section contained three series (Arribalzagia, Myzorhynchus and Christya), while the Angusticorn Section contained the Cycloleppteron, Lophoscelomyia and Anopheles Series. The structure proposed by Reid and Knight (1961) was mainly retained in the current classification, except for *Christya*'s subsequent elevation to subgeneric rank. Therefore, the subgenus *Anopheles* currently consists of two sections and five series.

Subgenus *Baimaia*

The monotypic subgenus *Baimaia* was recently established by Harbach *et al*. (2005) for a species occurring in Southeast Asia, and has subsequently remained a subgenus without subdivisions.

Subgenus *Cellia*

Theobald (1902) divided Anophelinae (Anophelina) into eight genera, including the novel genera *Cellia*, *Grassia* and *Howardina*. Theobald (1907, 1910) also described three additional genera, *Neocellia*, *Neomyzomyia* and *Pseudomyzomyia*. Some of these generic names were already in use and Blanchard (1902) proposed *Pyretophorus* and *Myzomyia* as replacement names for *Howardina* and *Grassia*, respectively. Edwards (1921) later recognised *Myzomyia* as a subgenus of *Anopheles* and Christophers (1924) divided *Myzomyia* into five groups (Cellia, Myzomyia, Neocellia, Neomyzomyia and Pseudomyzomyia). Edwards (1932) subsequently replaced Pseudomyzomyia with Pyretophorus, while Christophers (1933) included Paramyzomyia (Christophers & Barraud 1931) as an additional group.

Gillies and De Meillon (1968) eventually considered *Cellia* a subgenus of *Anopheles* and listed six series within the subgenus (Cellia, Myzomyia, Neocellia, Neomyzomyia, Pyretophorus and Paramyzomyia). The authors also divided Neomyzomyia and Myzomyia into three and four sections, respectively, and recognised two sections within Paramyzomyia. Currently, the subgenus *Cellia* retains the six series listed by Gillies and De Meillon (1968).

Subgenus *Christya*

Theobald (1903d) originally established *Christya* as a genus. It was subsequently listed as a group within the subgenus *Anopheles* by Christophers (1924) and later as a series within the same subgenus (Reid & Knight 1961). *Christya* was finally elevated to subgeneric rank by Harbach and Kitching (2016).

Subgenus *Kerteszia*

Kerteszia was initially described as a genus by Theobald (1905a). This taxon was later listed as a group within the subgenus *Nyssorhynchus* (Christophers 1924) and finally as a subgenus of *Anopheles* (Komp 1937). Its status as a subgenus of *Anopheles* has been retained in the current classification.

Subgenus *Lophopodomyia*

Lophopodomyia was erected as a subgenus of *Anopheles* by Antunes (1937) and has persisted as a subgenus since its description.

Subgenus *Nyssorhynchus*

Theobald's (1902) division of Anophelinae included the novel genus *Laverania* as one of its constituents, which was later renamed to *Nyssorhynchus* by Blanchard (1902). This was followed by Theobald's (1907) description of another relevant genus, *Myzorhynchella*. Subsequent authors considered *Nyssorhynchus* as a subgenus of *Anopheles* (Alcock 1911, Edwards 1911, Walker & Barbee 1914) and regarded Myzorhynchella as a group. Root (1923) adjusted the structure of *Nyssorhynchus* and synonymised the Myzorhynchella Group with the Nyssorhynchus Group, while Edwards (1932) once again listed Nyssorhynchus and Myzorhynchella as separate taxa. Edwards (1932) also divided the Nyssorhynchus Group into three series, including the Argyritarsis Series.

Two authors subsequently established many of the current subdivisions of *Nyssorhynchus*. Faran (1980) divided the subgenus into two sections (Albimanus and Argyritarsis) and split the Albimanus Section into two groups (Albimanus and Oswaldoi). Linthicum (1988) similarly divided the Argyritarsis Section into two groups (Argyritarsis and Albitarsis). Therefore, the current classification includes Albimanus, Argyritarsis and Myzorhynchella as sections within *Nyssorhynchus*, which are subdivided into the Albimanus, Albitarsis, Argyritarsis and Oswaldoi Series, instead of groups.

Subgenus *Stethomyia*

Stethomyia was described as an anopheline genus by Theobald (1902) and was subsequently considered a subgenus of *Anopheles* by Edwards (1932) and Christophers (1933). Its subgeneric status was retained in the current classification.

1.5 CONCLUSION

Culicidae has received considerable taxonomic attention throughout the last century due to its medical and veterinary significance. The structure of the family underwent countless adjustments throughout its taxonomic history and is still being refined, partly due to disagreements on certain taxonomic boundaries. However, despite the field's challenges, researchers now have the opportunity to gain novel insights into the affiliations of taxa with the implementation of a molecular approach. Since the classification of mosquitoes has traditionally been based on morphological data, there is still much to unearth regarding the true relationships within Culicidae.

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CHAPTER 2 – MATERIALS AND METHODS

2.1 SAMPLING

2.1.1 SAMPLING SITE SELECTION

The Free State Province is primarily situated within the Grassland Biome, with additional patches of the Savanna and Nama-Karoo Biomes occurring at the northern and western borders of the province (Mucina & Rutherford 2006). Mucina and Rutherford (2006) subdivided these biomes into bioregions and vegetation types, where such ecological units serve as a foundation for mosquito biodiversity. The distribution of vegetation can affect the diversity, presence and abundance of specific mosquito species (Brown *et al*. 2008, Amarasinghe & Dalpadado 2014, Roiz *et al.* 2015). Therefore, sampling sites were selected to represent a wide range of microhabitats in geographically distinct regions of the province, which varied in breeding opportunities, host availability and the degree of habitat disturbance. These sites ranged from the relatively pristine nature reserves to urban centres with substantial anthropological activity.

The urban and semi-urban sites provided the opportunity to sample species commonly associated with anthropological activity, including species that utilise artificial structures as breeding sites or species that are anthropophilic. Furthermore, smallholdings and farms provided the opportunity to sample zoophilic species that are commonly associated with livestock, especially species of veterinary importance. Finally, semi-pristine and pristine sites provided insight into the structure and abundance of natural mosquito communities, since these sites were relatively unaffected by anthropological activity and generally contained populations of indigenous vertebrate hosts. Within these sites, sampling was conducted near potential breeding and resting sites, which consisted of marshes, dams, streams, ponds, grasslands and dense vegetation.

2.1.2 SAMPLING PERMITS AND ETHICAL CLEARANCE

The necessary clearances were acquired from all appropriate officials prior to sampling. Ethical clearance was obtained from the Environmental & Biosafety Research Ethics Committee of the University of the Free State (Ethical clearance number UFS-ESD2018/0004) (Appendix 1). Sampling permits were requested in terms of the national environmental management: biodiversity act (10 of 2004) (Threatened or protected species regulations) and in terms of the Free State Nature conservation ordinance (8 of 1969) (Permit numbers JM 5040/2018 and JM5040/2019) (Appendix 2). This project also gained permission to conduct research in terms of Section 20 of the Animal Diseases Act, 1984 (Act No. 35 of 1984) (Project number UFS-ESD2018/0004) (Appendix 3), and all private property owners were consulted prior to sampling.

2.1.3 SAMPLING SITES

Sampling was conducted across 24 sampling sites in order to survey as many distinct geographical locations as possible, which were each classified as one of seven categories (Fig. 2.1 & 2.2; Table 2.1):

- Urban localities were defined as residential sites with a high degree of anthropological activity and a general absence of natural habitats in the vicinity (Fig. 2.2 a-e).
- Semi-urban localities were defined as sites with a moderate degree of anthropological activity, near large patches of undisturbed grasslands (Fig. 2.2 f-h).
- Rural sites were situated outside urban centres, adjacent to agricultural activity (Fig. 2.2 i & j).
- Smallholdings were located outside major urban centres, near low-intensity agricultural activity (animal husbandry) (Fig. 2.2 k-m).
- Farms were defined as sites consisting of a large area of land that was consistently utilised for livestock rearing or crop cultivation (Fig. 2.2 n).
- Semi-pristine sites were situated within large patches of grassland with reasonably natural faunal and floral communities, adjacent to urban activity (Fig. 2.2 o-q).
- Finally, pristine sites consisted of nature reserves with primarily natural faunal and floral communities (Fig. 2.2 r-u).

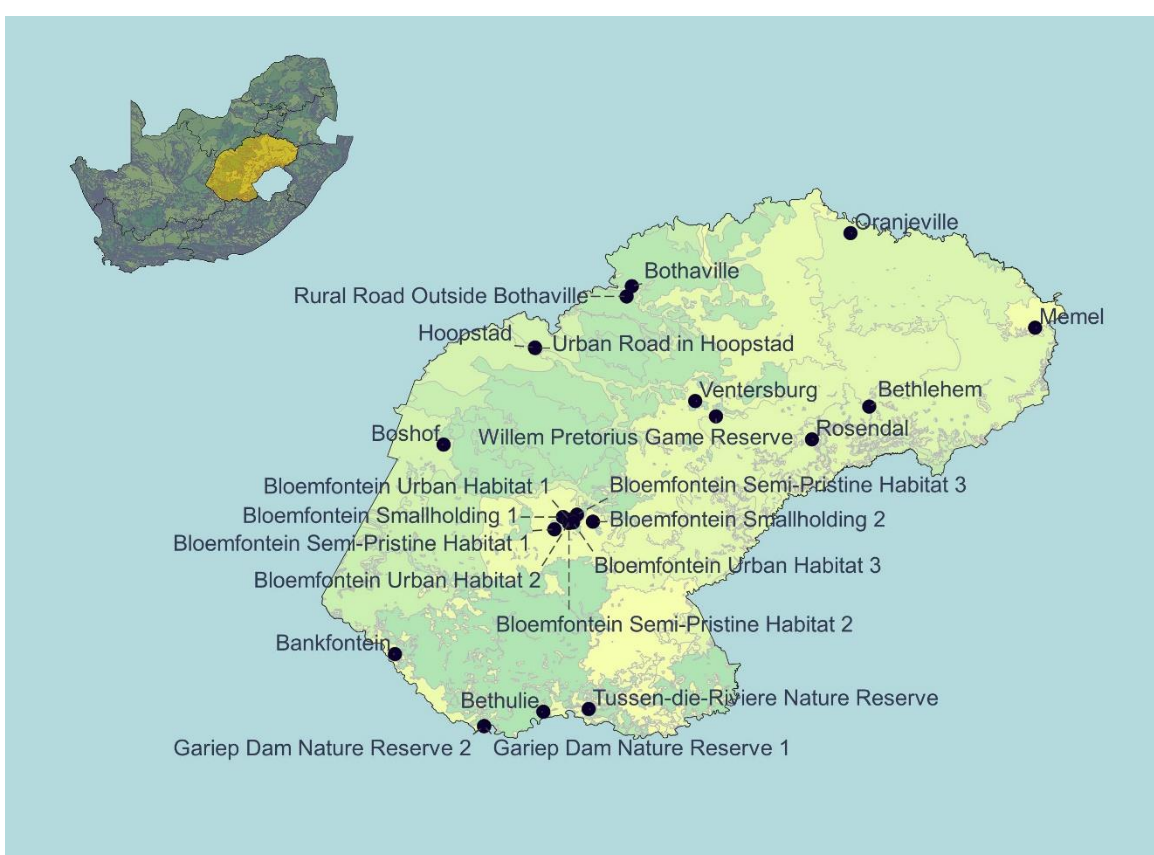

Fig. 2.1. Sampling site localities within the Free State Province, South Africa.

Table 2.1. Coordinates and habitat descriptions of the sampling sites.

SAMPLING SITE	COORDINATES	HABITAT TYPE	ELEVATION ABOVE SEA LEVEL	VEGETATION TYPE (MUCINA & RUTHERFORD 2006)
Bethulie	30°29'37.8"S 25°58'04.8"E	Urban	1,303 m	Xhariep Karroid Grassland
Bloemfontein Urban Habitat 1 (Langenhoven Park)	29°05'23.5"S 26°09'23.4"E	Urban	1,419 m	Winburg Grassy Shrubland
Bloemfontein Urban Habitat 2 (Langenhoven Park)	29°05'59.7"S 26°09'42.6"E	Urban	1,430 m	Bloemfontein Dry Grassland
Bloemfontein Urban Habitat 3 (Park West)	29°06'37.8"S 26°11'05.4"E	Urban	1,435 m	Winburg Grassy Shrubland
Boshof	28°32'28.8"S 25°14'19.5"E	Urban	1,257 m	Kimberley Thornveld
Bothaville	27°23'00.8"S 26°36'53.9"E	Urban	1,281 m	Vaal-Vet Sandy Grassland
Hoopstad	27°49'53.8"S 25°54'23.6"E	Urban	1,255 m	Kimberley Thornveld
Urban Road in Hoopstad	27°50'07.7"S 25°54'27.6"E	Urban	1,251 m	Highveld Alluvial Vegetation
Memel	27°41'13.7"S 29°33'46.2"E	Semi-urban	1,742 m	Amersfoort Highveld Clay Grassland
Oranjeville	26°59'44.4"S 28°12'47.3"E	Semi-urban	1,498 m	Frankfort Highveld Grassland
Rosendal	28°30'12.1"S 27°55'53.3"E	Semi-urban	1,697 m	Eastern Free State Sandy Grassland
Rural Road Outside Bothaville	27°27'30.0"S 26°34'35.8"E	Rural	1,320 m	Vaal-Vet Sandy Grassland
Ventersburg	28°13'21.4"S 27°04'41.0"E	Rural	1,335 m	Central Free State Grassland
Bethlehem	28°15'49.5"S 28°21'00.0"E	Smallholding	1,687 m	Eastern Free State Sandy Grassland
Bloemfontein Smallholding 1 (Tanbryn Small Holdings)	29°04'15.0"S 26°06'40.0"E	Smallholding	1,362 m	Bloemfontein Dry Grassland
Bloemfontein Smallholding 2 (Roodewal Small Holdings)	29°06'17.2"S 26°19'48.6"E	Smallholding	1,333 m	Bloemfontein Dry Grassland
Bankfontein	30°04'21.2"S 24°52'59.9"E	Farm	1,188 m	Besemkaree Koppies Shrubland
Bloemfontein Semi-Pristine Habitat 1 (Kloofeind)	29°09'37.4"S 26°02'56.5"E	Semi-pristine	1,429 m	Winburg Grassy Shrubland
Bloemfontein Semi-Pristine Habitat 2 (eKaru)	29°06'53.3"S 26°09'17.7"E	Semi-pristine	1,411 m	Bloemfontein Dry Grassland
Bloemfontein Semi-Pristine Habitat 3 (The Kloof)	29°03'11.3"S 26°12'50.8"E	Semi-pristine	1,379 m	Winburg Grassy Shrubland
Gariep Dam Nature Reserve Location 1	30°35'54.5"S 25°32'08.0"E	Pristine	1,268 m	Besemkaree Koppies Shrubland
Gariep Dam Nature Reserve Location 2	30°35'57.6"S 25°32'01.1"E	Pristine	1,267 m	Besemkaree Koppies Shrubland
Tussen-die-Riviere Nature Reserve	30°28'26.6"S 26°17'58.4"E	Pristine	1,437 m	Besemkaree Koppies Shrubland
Willem Pretorius Game Reserve	28°20'06.0"S 27°13'47.4"E	Pristine	1,378 m	Central Free State Grassland

Fig. 2.2. Images of several sampling sites: a) Bloemfontein Urban Habitat 1; b) Bloemfontein Urban Habitat 2; c) Boshof; d) Bothaville; e) Hoopstad; f) Memel; g) Oranjeville; h) Rosendal; i) Rural Road Outside Bothaville; j) Ventersburg; k) Bethlehem; l) Bloemfontein Smallholding 1; m) Bloemfontein Smallholding 2; n) Bankfontein; o) Bloemfontein Semi-Pristine Habitat 1; p) Bloemfontein Semi-Pristine Habitat 2; q) Bloemfontein Semi-Pristine Habitat 3; r) Gariep Dam Nature Reserve Location 1; s) Gariep Dam Nature Reserve Location 2; t) Tussen-die-Riviere Nature Reserve; u) Willem Pretorius Game Reserve.

2.1.4 SAMPLING PERIOD

Sampling was primarily conducted during the spring and summer months to coincide with the peak in mosquito abundance across various sampling sites. Rainy and windy sampling conditions were avoided, since wind reduces mosquito host-seeking behaviour due to their weak flying capacity (Service 1980). Most localities were represented by a single sampling session between 14 November 2018 and 14 April 2020, while multiple sampling efforts were undertaken at Bloemfontein semi-pristine habitat 2, Bloemfontein urban habitat 1, Bloemfontein urban habitat 2, Bloemfontein urban habitat 3 and Willem Pretorius Game Reserve. Within the sampling sites, mosquito presence and abundance may have been subject to numerous biotic and abiotic factors, and dependent on the local environmental dynamics (Pliego Pliego *et al.* 2017, Ewing *et al.* 2019, Mayi *et al.* 2020). Therefore, certain species within a region may have been overlooked due to the timing of the sampling sessions. However, the span of the sampling period still coincided with the activity of numerous species within the region. Active sampling methods (sweep netting and hand collection) were used whenever mosquitoes were observed, usually between 5 PM and 12 AM, while traps were erected at 5 PM and collected at 6 AM the following morning.

2.1.5 SAMPLING METHODS

Various sampling methods were employed to sample a diverse array of species. This included a carbon dioxide (CO_2) baited net, a CO_2 baited suction trap based on the CDC light trap (Sudia & Chamberlain 1962), hand collection and sweep netting. Since mosquito females use CO_2 as a cue during host location (Gillies 1980), it was an effective bait for many mosquito species.

The CO_2 baited net (Fig. 2.3 a) was recommended by researchers at the NICD Vector Control Reference Laboratory and consisted of a suspended cubic net (1.5 m x 1.5 m x 2 m), with the bottom plane open and suspended 0.5 m above the ground. A Styrofoam container with a punctured lid containing 2.5 kg dry ice (CO_2) was placed at the centre of the net during each sampling session, to serve as an attractant. The exposed base allowed mosquitoes to enter the trap and become confined as they flew upwards, searching for a host. This trap was erected in the early evening and all mosquitoes were collected at sunrise the following morning with an aspirator. The second baited method, a suction trap (Fig. 2.3 b), consisted of a small battery-operated fan that drew bypassing mosquitoes into a collection net. A container with dry ice (CO_2) was connected to the trap to serve as an attractant in addition to the trap's UV light. The suction trap was also operated from the early evening to the following morning.

Fig. 2.3. The two CO_2 baited traps used during sampling: a) The CO_2 baited net; b) The CO_2 baited suction trap.

To counter the female bias of the CO_2 baited traps, sweep netting and hand collection were also employed. Here, sweep netting was used to collect individuals within mating swarms and hand collection was used whenever individual mosquitoes were accessible in the vicinity.

Since large numbers of mosquitoes were often collected at each site, mosquitoes were collectively euthanised before the specimens were individually processed. Specimens were collected in a sealed plastic bag and placed in a cold environment (either a freezer or a dry ice container) until the mosquitoes were no longer active. The euthanised mosquitoes were then transported to the University of the Free State and stored at -80°C to preserve the integrity of their DNA.

2.2 SPECIMEN AND DATA PROCESSING

2.2.1 SPECIMEN IDENTIFICATION AND PROCESSING

Individual mosquitoes were placed into labelled sterile 1.5 ml microcentrifuge tubes, where the specimen codes were linked to the relevant sampling data on a Microsoft Excel spreadsheet. All instruments (Petri dishes, pincettes and pins) were frequently sterilised with a 10% liquid bleach solution and wiped with a 70% ethanol solution to minimise DNA contamination.

The specimens were viewed under a Nikon SMZ445 stereo microscope with an Olympus KL1600 LED light source. Individual mosquitoes were identified to species or complex level with the keys provided by Jupp (1996) for culicines, and Gillies and Coetzee (1987) for anophelines. The identities of non-typical and slightly damaged specimens were corroborated with the keys of Muspratt (1955), Huang (2001), Highton (1983) and McIntosh (1973 & 1975), since the authors often used different features for species identifications. Each specimen's relevant information was recorded, including instances of intraspecific variation. Representative specimens of each species and morphotype were selected for DNA sequencing. The selected specimens were photographed bilaterally, dorsally and ventrally with a

Canon 5D Mark IV fitted with a Sigma 150 mm macro lens and Kenco extensions. The photos were then processed in Helicon Focus (Helicon Soft Limited) to generate high-quality stacked images for each specimen.

Since only a tiny amount of input DNA was required for the analyses, either the legs or heads of the specimens were used as a source of DNA. The sample material was carefully separated from the mosquito with sterilised pins and screened for evident contaminants, mainly consisting of loose scales from other mosquitoes. These contaminants were carefully removed with a sterilised paintbrush and the decontaminated samples were individually placed into autoclaved 1.5 ml microcentrifuge tubes.

2.2.2 DATA PROCESSING

The recorded data for each sampled specimen consisted of the associated sampling methods; collection date, time and location; associated habitat and vegetation types; specimen species, sex and condition; instances of morphological variation; and a unique specimen identifier. The relevant data were processed with Microsoft Excel's pivot chart function and the applicable charts and tables were generated from the subsets of data.

The generated distribution data of sampled specimens were imported into QGIS 3.18 and combined with all accessible historical records of sampled species within the Free State Province, as listed in Table 3.3. The compiled dataset was used to generate distributions maps of the sampled mosquito species within the region.

2.3 DNA ANALYSES

2.3.1 DNA EXTRACTION

As required by the stipulations of the Section 20 permit, extraction was performed in a Biosafety Level 2 laboratory within a biosafety cabinet. Here, a disposable pestle was inserted into each microcentrifuge tube along with the sample. The bottom of the tube was submerged in liquid nitrogen for about 30 seconds, which facilitated the homogenisation of specimens into a fine powder. Extraction was performed with the DNeasy® Blood & Tissue Kit (Qiagen, cat. no. 69504), where the procedure mainly adhered to the standard protocol, except for a few minor adjustments. This included the additional homogenisation of specimens after the addition of 180 µl Buffer ATL to improve cell lysis. The liquid also facilitated the removal of sample residue on the pestle. The specimens were lysed overnight and eluted in 50 µl Buffer AE instead of the recommended 200 µl to increase the DNA concentration.

2.3.2 DNA AMPLIFICATION

The DNA was amplified with the polymerase chain reaction (PCR) reagents listed in Table 2.2 for downstream applications. Each PCR session's reagents were prepared as a master mixture containing all the necessary components for increased accuracy. This master mixture was divided into individual reaction tubes containing the DNA template. Each reaction consisted of 0.25 DNA polymerase, 1 µl dNTPs, 10 µl buffer, 5 µl $MgCl_2$, 2 µl forward primer, 2 µl reverse primer, 2 µl DNA template and 27.75 µl nuclease-free water with a total reaction volume of 50 µl.

The COI region was amplified with the primer pair listed in Folmer *et al.* (1994); LCO1490: 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' and HCO2198: 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3', the ITS2 region with the primers used by Djadid *et al.* (2007); 5.8 s: 5'-ATC ACT CGG CTC GTG GAT CG-3' and 28 s: 5'-ATG CTT AAA TTT AGG GGG TAG TC-3' and the domain 1 (D1) portion of the 28S large ribosomal subunit RNA gene with the primers provided by Friedrich & Tautz (1997); DIF: 5'-CCC (C/G)CG TAA (T/C)TT AAG CAT AT-3' and DIR: 5'-ACT CTC TAT TCA (A/G)AG TTC TTT (G/C)-3'.

DNA from the samples was amplified with a T100™ Thermal Cycler (Bio-Rad) and a gradient PCR was performed to optimise the annealing temperatures. The ideal temperatures were 45°C, 52°C and 44°C for the COI, ITS2 and 28S primers, respectively. Thereafter, the following cycling conditions were used: initial denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation (95°C for 30 seconds), annealing (optimised temperature per primer pairs for 30 seconds) and extension (72°C for 1 minute), and concluded with a final extension for 5 minutes at 72°C.

Table 2.2. Reagents used in the polymerase chain reaction.

PCR COMPONENT	PRODUCT NAME	MANUFACTURER	CATALOGUE NUMBER	CONCENTRATION
DNA Polymerase	GoTaq® Hot Start Polymerase / GoTaq® G2 Hot Start Taq Polymerase	Promega	M5001, M7401	5 u / µl
dNTP	Deoxynucleotide (dNTP) Solution Mix	New England Biolabs® (UK) Ltd	N0447S	10 mM of each dNTP
Buffer	5X Green GoTaq® Flexi Buffer	Promega	M8911	-
$MgCl_2$	Magnesium chloride	Promega	A3511	25 mM
Water	Nuclease-free water	Invitrogen™	AM9937	-

2.3.3 GEL ELECTROPHORESIS AND PCR CLEAN-UP

The products of PCR amplification were surveyed with agarose gel electrophoresis. Depending on their availability, one of two different molecular markers were used as a reference for amplicon length, which consisted of the GeneRuler DNA Ladder Mix (Thermo Scientific™, cat. no. SM0331) or the PCRBIO Ladder II (PCR Biosystems, cat. no. PB40.12-05). A 1:100 GelRed® Nucleic Acid Gel Stain solution (Millipore, cat. no. SCT123) was prepared for DNA fluorescence and 1 µl of this solution was mixed with 5 µl of the PCR product. This mixture was then loaded onto a 1% agarose gel and electrophoresed

at 100 V until sufficient separation of the bands were observed. The processed samples were visualised with a Bio-Rad Gel Doc EZ Imaging System. DNA bands were classified according to their relative brightness (bright, medium or faint), which functioned as an estimate of the DNA concentration. The successful PCR products were then purified with the Wizard® SV Gel and PCR Clean-Up System (Promega, cat. no. A9281).

2.3.4 SANGER SEQUENCING

Cycle sequencing was performed with the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™, cat. no. 4337455). The reagents were prepared as master mixes and divided into individual reaction tubes, which contained all the necessary reagents for the forward and reverse reactions. The purified amplicon was added to each reaction mix and the amount was adjusted according to the amplicon's relative brightness during gel visualisation (bright – 3 µl; medium – 4 µl; faint – 6 µl). Each reaction consisted of 2 µl BigDye™ Terminator v3.1 Ready Reaction Mix, 1 µl 5X sequencing buffer, 0.32 µl primer (forward or reverse), 3-6 µl template DNA and nuclease-free water to a total reaction volume of 10 µl. The reaction was executed with the following cycling conditions: initial denaturation at 96°C for 1 minute, followed by 25 cycles of denaturation (96°C for 10 seconds), annealing (50°C for 5 seconds) and extension (60°C for 4 minutes). Samples were then purified with the BigDye XTerminator™ Purification Kit (Applied Biosystems™, cat. no. 4376486) and the products were submitted to the University of the Free State's Department of Genetics for sequencing.

2.4 PREPARATION FOR PHYLOGENETIC ANALYSES

2.4.1 SEQUENCE GENERATION AND IDENTITY VERIFICATION

The University of the Free State's Department of Genetics provided the sequencing results as electropherograms, which were used to generate consensus sequences in SeqTrace (Stucky 2012). The forward and reverse sequences from each sample were processed and aligned, and discrepancies between the two sequences were manually screened and corrected. Any base uncertainties were indicated by ambiguous nucleotides (N).

The final generated sequences were screened against the GenBank database to identify potentially misidentified sampled specimens. The query was performed with the standard nucleotide BLAST function on the NCBI platform (National Center for Biotechnology Information 2020). Most queries were submitted with default parameters, which included the megablast optimisation program for highly similar sequences. Considering the prevalence of misidentified specimens on DNA repositories, the consensus of top identity matches were taken into account, rather than specific identity matches. A few sequences yielded no meaningful matches with the megablast function, and therefore the blastn optimisation program for somewhat similar sequences was selected. The results were arranged according to the highest percentage identities and lowest E-values (match significance). The top identity matches were

noted for all sequenced specimens, while the results corresponding to the lowest E-value provided a more appropriate match for a few specimens.

Priority was given to the species matches provided by the barcoding region (COI), unless this region was not successfully sequenced. In such cases, matches provided by the ITS2 and 28S results were considered. The specimens belonging to species that were represented within the GenBank database, yet did not produce a close conspecific match were morphologically reidentified. The generated sequences were then added to the datasets and uploaded to BOLD (Molecular Phylogeny of South African Mosquitoes; MPSAM001-21 - MPSAM062-21) for public accessibility.

2.4.2 TAXON SAMPLING

Datasets were constructed to include as many major clades (section, series and groups) and South African species as possible, to conduct comprehensive phylogenetic analyses of the three target DNA regions. The species coverage depended on the sequence availability across the three selected DNA regions, since relatively few sequences were available for the 28S D1 domain. Additionally, very few database voucher specimens were represented by multiple target sequences, and it was therefore not possible to construct comprehensive datasets with combined sequence data for multiple DNA regions. Furthermore, South African species were also poorly represented within the public DNA databases. The limited coverage of these database sequences consequently limited the scope of the current phylogenetic investigations. Therefore, the aim was rather to ensure that the represented species were shared between the COI, ITS2 and 28S datasets as far as possible, since independent datasets were constructed for each DNA region and genus.

DNA sequences for the representative taxa and target DNA regions were obtained from GenBank (Benson *et al.* 2012) and BOLD (Ratnasingham & Hebert 2007). A search for sequences originating from type material was performed with a Taxonomy Entrez query as specified by Federhen (2015), which could not locate any type sequence data for mosquitoes. Therefore, to ensure that database sequences were ascribed to the correct species, sequences from three unique sets of authors were included for each representative species, where possible. Since relatively few target sequences were linked to publications, unpublished sequences were also included to ensure coverage for the major taxonomic divisions within each genus. The D1 domain of the 28S region was sequenced in the current study and homologous database sequences were required for the datasets. Within GenBank, it was often not specified whether 28S sequences contained the D1 domain. Therefore, relevant database sequences were located with a BLAST search (Altschul *et al.* 1990), where the sequences of sampled specimens were entered as a query with default parameters. All applicable matching sequences were then included in the multiple sequence alignment (MSA).

The taxon coverage across the three DNA regions was also dependent on the structure of the genus and the relevance of its subdivisions to South Africa. For *Aedes*, representative species were selected

for all available subgenera, while additional superspecific representatives were included for two subgenera (*Ochlerotatus* and *Stegomyia*) due to their relatively large size. Furthermore, all available South African *Aedes* species listed in Jupp (1996) were added to the dataset. For *Culex* and *Anopheles*, representatives were selected for all available subgenera, sections, series and groups. All available South African species listed in Jupp (1996) and Gillies & Coetzee (1987) were also included in the datasets. Here, groups containing South African species were further represented by species from each subgroup and complex, where possible.

Outgroups were selected to provide the most meaningful phylogenetic comparisons, since the inclusion of sister clades generally results in improved tree rooting (Smith 1994). The selection was guided by sequence availability and the taxon's recurrent affiliation with the ingroup, as recovered by multiple morphological and molecular analyses (Harbach & Kitching 1998, Isoe 2000, Shepard *et al.* 2006, Harbach 2007). The selected outgroup genera consisted of *Armigeres, Culiseta* and *Toxorhynchites* for *Aedes*, *Culex* and *Anopheles*, respectively. Here, each dataset incorporated two outgroup sequences, representing two different species, where possible.

2.4.3 MULTIPLE SEQUENCE ALIGNMENT

All generated and database sequences (Appendix 4) were imported into MEGA X (Kumar *et al.* 2018) and processed to generate a FASTA file. Sequences were aligned with the MAFFT version 7 online platform (Katoh *et al.* 2019) and settings were adjusted for each DNA region. The COI and 28S regions were aligned using the G-INS-i strategy with a 1PAM / K = 2 scoring matrix, a gap opening penalty of 1 and default settings for the remaining parameters. Katoh and Toh (2008) proposed the iterative refinement method, G-INS-i, for small-scale alignments with a global homologous structure, appropriate for the COI and 28S datasets. The web interface also recommended a 1PAM / K = 2 scoring matrix for closely related sequences, suitable for the current single-genus datasets. Finally, the gap opening penalty was reduced to the lowest setting (1) to indicate the presence of probable gaps. All generated alignments were then manually inspected and corrected if necessary.

Since the ITS2 region comprised many indels, automatic alignment approaches often yielded erroneous results. The exclusion of these ambiguously aligned sections also resulted in the major loss of phylogenetic resolution. Therefore, numerous alignment algorithms were tested, including MUSCLE (Edgar 2004), CLUSTAL W (Thompson *et al.* 1994) and methods guided by the secondary RNA structure, such as LocARNA (Smith *et al.* 2010) and MAFFT's Q-INS-i option. Multiple MAFFT settings were also examined and the E-INS-i strategy yielded the most coherent results when inspected. However, due to the scale and complexity of the ITS2 region, the alignment was not manually adjusted.

Individual sequences displaying an apparent lack of homology to the target region were removed, replaced by conspecific sequences and realigned. Any base discrepancies between the generated sequences and those belonging to conspecific database specimens were verified in the original

electropherograms. The final MSA was processed in BioEdit (Hall 1999) and trimmed to contain the target region, where multiple annotated GenBank sequences were used as a reference. Any missing data were also replaced with question marks to differentiate between true gaps and undetermined sites. The MSA was finalised in Mesquite (Maddison 2008), which included the truncation of taxon names and removal of prohibited characters that would interfere with the phylogenetic analyses. The alignments were then converted to the appropriate formats, including NEXUS files for the Bayesian analyses and PHYLIP files for the maximum likelihood analyses.

2.5 PHYLOGENETIC ANALYSES

2.5.1 MAXIMUM LIKELIHOOD AND BAYESIAN INFERENCE OF PHYLOGENY

Maximum likelihood and Bayesian phylogenetic analyses were executed for each of the three genera and gene regions with the University of the Free State's High-Performance Computing resources. The Bayesian analyses were conducted with MrBayes version 3.2.3 (Huelsenbeck & Ronquist 2001) and the parallel version of its Metropolis-coupled Markov chain Monte Carlo capabilities (Altekar *et al.* 2004).

Since the prevalence of misidentified and poor quality sequences in public sequence databases has been well-documented (Bridge *et al.* 2003, Nilsson *et al*. 2006, Valkiūnas *et al*. 2008), preliminary Bayesian analyses were conducted to identify potentially misidentified database specimens. The parameters were set to the mixed substitution option (nst = mixed), which permitted sampling across various substitution models and circumvented the need for *a priori* model testing. Furthermore, a proportion of invariable sites (rates = invgamma) was selected, since the datasets consisted of a wide range of intrageneric taxa that robustly displayed numerous invariable sites. The Markov chain was sampled every 500 generations (samplefreq = 500) and a burnin percentage of 25% was selected. The outgroup was specified and the analysis was executed with a total of 2,000,000 generations for each of the two runs, which consisted of four chains each. Since species sequences often originated from three separate sets of authors, conspecific clades usually represented corroborated species identities. The trees were examined for specimens with highly aberrant placements, which likely represented misidentified specimens. The potentially misidentified sequences were replaced with conspecific sequences within the MSA in order to increase the phylogenetic utility of the species-level clades.

The final Bayesian analyses were then executed once the datasets produced relatively coherent species-level clades. These analyses used the same parameters as the preliminary analyses (nst = mixed; rates = invgamma; samplefreq = 500; burnin percentage of 25%; outgroups were specified), except for the increased number of generations (20,000,000) and runs (n = 3). The temperature was also uniquely optimised for each analysis until sufficient chain swapping was achieved.

The maximum likelihood analyses were based on the final dataset and were executed with RAxML version 8.2.12 (Stamatakis 2014), since it permitted data partitioning according to the codon positions,

as in MrBayes. The GTR and GAMMA models were selected (-m GTRGAMMA), which allowed partition-specific estimations of the alpha values, substitution rates, base frequencies and branch lengths (-M). Furthermore, the parsimony inferences and rapid bootstrapping were executed with the same seed (-p 12345; -f a –x 12345). Finally, the outgroup was specified and 1,000 bootstrap replicates were conducted. Here, GTRGAMMAi was avoided despite the presence of invariable sites in the MSA. The RAxML developer (Stamatakis 2016) raised concerns over the interaction of the alpha and P-Invar parameters and stated that GTRGAMMA should essentially correct for the rate heterogeneity of invariable sites.

Since the availability of sequence data was limited across the various target DNA regions, it was not possible to construct a consolidated dataset for the included species and analyses were conducted independently for each genus and DNA region. The settings for MrBayes and RAxML were consistent across the three DNA regions, except for the inclusion of partitions for the COI datasets. The three codon positions of protein-coding regions have unique evolutionary constraints and evolve at different rates (Bofkin & Goldman 2007). Bofkin and Goldman (2007) therefore recommended the partitioning of individual codon positions, where model parameters are independently estimated for each partition to reflect the unique evolution of these sites. Since the target region of the COI datasets was situated within the gene's coding region, the datasets were divided into three partitions, with each consisting solely of the first-, second- or third-codon position's nucleotides. The substitution rates, base frequencies, alpha values and proportion of invariable sites were estimated independently for each of the three COI codon position partitions in both the Bayesian and maximum likelihood analyses. However, since the target ITS2 and 28S regions did not consist of protein-coding sequences, the variables were estimated for a single partition consisting of the entire dataset.

2.5.2 PHYLOGENETIC DEPICTION

Phylogenetic trees were constructed for each of the three genera, gene regions and phylogenetic methods, yielding a total of 18 phylogenetic trees (Appendix 5). The generated trees were initially processed with FigTree (Rambaut 2009) and then augmented in Adobe Photoshop, where the results from the two phylogenetic methods were condensed into a single tree. Here, the support values of the clades shared between the two phylogenetic methods were superimposed onto the maximum likelihood topology. Clades were also annotated with the applicable taxonomic subdivisions, including sections, series, groups, subgroups and complexes. Branches with bootstrap support values (BS) of ≥70% and posterior probabilities (PP) of ≥95% were considered to be significantly supported, based on the thresholds employed by many authors in Bayesian and maximum likelihood phylogenies (Leaché & Reeder 2002, Quenouille *et al*. 2003, Harris *et al*. 2004, Miller *et al*. 2004, Vinuesa *et al*. 2005, Jiang *et al*. 2006, Sung *et al*. 2007, Schuettpelz & Pryer 2007, Hua *et al*. 2016).

2.6 HAPLOTYPE COMPARISONS

To date, few genetic investigations have examined the relationships of South African mosquitoes. Therefore, their relationship towards presumed conspecific specimens have often not been examined from a molecular perspective, and the recovery of non-monophyletic species-level clades would impede the phylogenetic interpretation of South African species relationships.

The utility of COI to reveal cryptic diversity within mosquito species (Bourke *et al.* 2021, Motoki *et al.* 2021) and the ability of ITS2 to distinguish between closely related species (Li *et al.* 2005, Manonmani *et al.* 2007) depends on the high-resolution signal engrained within the DNA data. These relatively fast evolving regions can provide insight into the taxonomic structure of species and reveal potential issues that obscure phylogenetic interpretations. The joint use of COI and ITS2 data can also reflect the geographical structuring of mosquito populations (Rosera *et al.* 2012), which plays a significant role in speciation events (Barraclough & Vogler 2000). Therefore, a substantial degree of genetic differentiation between specimens from distinct geographical regions may justify investigations into their taxonomic status.

The COI and ITS2 haplotypes of sampled species were examined for shared and unique alleles to gain insight into the phylogenetic placements of sampled specimens and their relationship with conspecific individuals. The datasets often consisted of conspecific sequences from several sets of authors, to provide a consensus on the identity of database sequences. The intraspecific polymorphic and indel sites were summarised in sequence tables to display the genetic structuring of these species. To achieve this, the MSAs were converted to dot plots with BioEdit (Hall 1999) and processed in Microsoft Excel to illustrate the various alleles. However, some datasets provided no comparable results, since the sampled species were often only represented by a single sequence or limited to a specific DNA region.

The haplotypes were manually screened for any alleles associated with specific geographical regions, which often influenced the phylogenetic placements and affected clade support. Despite the relatively small sample sizes, polymorphic sites and clusters of alleles still provided insight into the relationships and genetic features of South African species in relation to non-South African conspecific specimens.

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CHAPTER 3 - RESULTS

3.1 SAMPLING RESULTS OVERVIEW

A total of 4,197 mosquitoes were sampled across the Free State Province. All sampled species belonged to one of five genera (*Aedes*, *Anopheles*, *Culex*, *Culiseta* and *Mansonia*), representing 11 subgenera and 26 sampled morphospecies (Table 3.1). The vast majority of sampled individuals belonged to *Aedes* (83%) and *Culex* (17%), while less than 1% of specimens belonged to *Anopheles*, *Culiseta* and *Mansonia* collectively. Most sampled specimens retained their sexual characteristics, which included 1,642 females, 2,388 males and 167 specimens that were too damaged to sex. Both the abundance of *Aedes* and the overall skewed sex ratio resulted from a single male-dominated mating swarm during sampling (Bloemfontein semi-pristine habitat 2). The Hoopstad locality also exhibited a substantially male-biased sex ratio (2:1), despite the use of a female-biased CO_2 baited net. Here, many males were observed in the vicinity and may have inadvertently entered the trap, or were attracted by the females already caught within the trap. Nonetheless, most localities produced either a balanced or female-biased sex ratio ranging from 1:1 to 1:132, which was expected for the CO_2 baited traps.

During morphological identifications, 22 morphospecies were successfully identified to species-level or as one of two closely related species (Fig. 3.1). This included a few sampled individuals with intermediate characteristics between *Cx*. (*Culex*) *pipiens* and *Cx*. (*Cux*.) *quinquefasciatus*. No immature morphological data was available for the sampled *An*. (*Cellia*) *cydippis* and *An*. (*Cel*.) *squamosus* specimens, which was necessary to differentiate between the two species (Coetzee 2020). Therefore, these individuals were listed as *Cx*. (*Cux*.) *pipiens* / *quinquefasciatus* and *An*. (*Cel*.) *cydippis* / *squamosus*, respectively.

The specific identity of the remaining four morphospecies was uncertain. Two of these specimens, *Ae*. (*Stg*.) cf. *ledgeri* and *Cx*. (*Cux*.) cf. *antennatus* were substantially damaged, and the best estimate of the species identity was given based on the available features. These species have also previously been recorded in South Africa. The remaining specimens, *Ae*. (*Ochlerotatus*) cf. *harrisoni* and *Cx*. (*Cux*.) cf. *neavei* displayed characteristics that departed from their current species descriptions. The *Cx*. (*Cux*.) cf. *neavei* specimen exhibited a dark dorsal line extending halfway down the hindfemur, as found in *Cx*. (*Cux*.) *neavei*, while simultaneously possessing a prominent longitudinal pale line on the midfemur as seen in *Cx*. (*Cux*.) *univittatus* (Jupp 1971, 1996). Finally, the uncertainty regarding the *Ae*. (*Och*.) cf. *harrisoni* specimens was caused by the variation in their thoracic setae. With the key of Jupp (1996), the specimens were identified as *Ae*. (*Och*.) *harrisoni*. However, the females only intermittently possessed lower mesanepimeral setae, which contradicted the description of Muspratt (1953). Since the identity of these four morphospecies could not be confirmed and the well-preserved specimens were not successfully sequenced, these specimens were omitted from further discussions.

Table 3.1. Identity of the 26 sampled morphospecies and the corresponding sampling results.

GENUS	SUBGENUS	SPECIES	SAMPLING METHOD	ABUNDANCE
Aedes	*Aedimorphus*	*Ae. dentatus*	CO_2 baited net	n = 268
		Ae. eritreae	CO_2 baited net	n = 12
		Ae. subdentatus	CO_2 baited net	n = 4
	Catageiomyia	*Ae. mixtus*	Suction trap	n = 2
	Coetzeemyia	*Ae. fryeri*	Hand collection CO_2 baited net	n = 1 n = 1
	Neomelaniconion	*Ae. luridus*	Sweep netting	n = 3
		Ae. luteolateralis	CO_2 baited net	n = 1
		Ae. mcintoshi	Hand collection CO_2 baited net Sweep netting	n = 1 n = 2 n = 34
		Ae. unidentatus	CO_2 baited net	n = 4
	Ochlerotatus	*Ae. caballus*	Hand collection Sweep netting	n = 21 n = 7
		Ae. juppi	Hand collection CO_2 baited net Sweep netting	n = 14 n = 4 n = 164
	Stegomyia	*Ae. aegypti*	Hand collection	n = 11
		Ae. contiguus	Hand collection	n = 1
Anopheles	*Cellia*	*An. cydippis / squamosus*	Suction trap CO_2 baited net	n = 2 n = 1
Culex	*Culex*	*Cx. pipiens*	Hand collection CO_2 baited net	n = 3 n = 90
		Cx. quinquefasciatus	Hand collection CO_2 baited net	n = 16 n = 6
		Cx. simpsoni	CO_2 baited net	n = 1
		Cx. theileri	Suction trap Hand collection CO_2 baited net	n = 6 n = 1 n = 135
		Cx. univittatus	Suction trap Hand collection CO_2 baited net Sweep netting	n = 7 n = 6 n = 112 n = 1
	Maillotia	*Cx. salisburiensis*	Hand collection	n = 1
Culiseta	*Allotheobaldia*	*Cs. longiareolata*	Hand collection CO_2 baited net	n = 4 n = 11
Mansonia	*Mansonioides*	*Ma. africana*	CO_2 baited net	n = 1
AMBIGUOUS IDENTIFICATIONS				
Aedes	*Ochlerotatus*	*Ae.* cf. *harrisoni*	CO_2 baited net	n = 12
	Stegomyia	*Ae.* cf. *ledgeri*	Hand collection	n = 1
Culex	*Culex*	*Cx.* cf. *antennatus*	Suction trap	n = 1
		Cx. cf. *neavei*	CO_2 baited net	n = 2

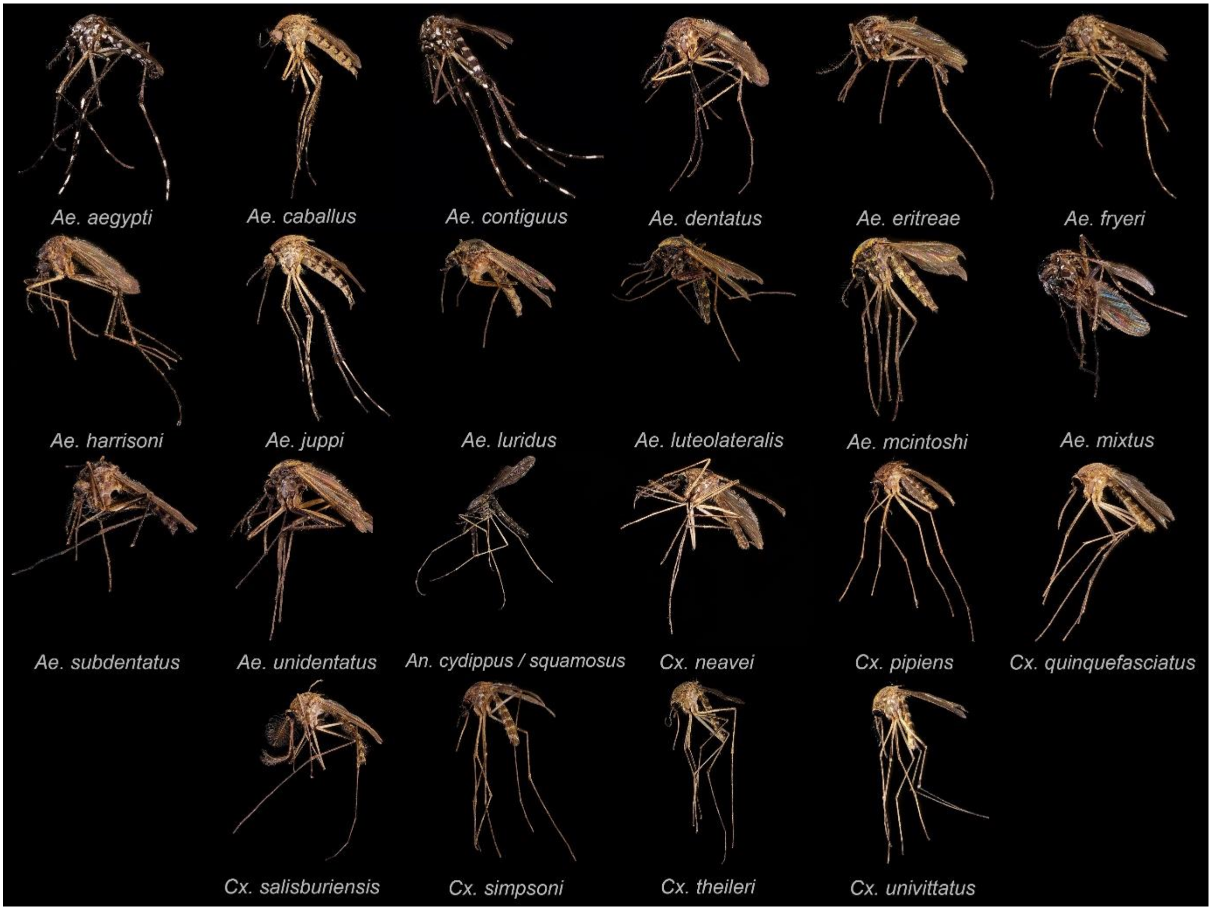


Fig. 3.1. Representatives of the 22 identified species (out of 26 morphospecies) sampled within the Free State Province.

3.2 SAMPLING METHODS

Various sampling methods were employed during the current study, which produced a diverse array of species. The majority of specimens were collected with sweep netting (n = 2,941) and the CO_2 baited net (n = 1,113), while the suction trap (n = 42) and hand collections (n = 39) produced a low abundance of specimens. Here, the efficacy of the sweep netting was due to a large mosquito swarm at a single locality. Sweep netting and hand collection were only employed at a few sites, yet several species were exclusively associated with these methods. A total of 12 species were sampled by hand, which was the only method that yielded *Ae*. (*Stg.*) *aegypti*, *Ae*. (*Stg.*) *contiguus* and *Cx*. (*Mai.*) *salisburiensis* specimens, while sweep netting recovered a total of five species and was uniquely associated with *Ae*. (*Neo.*) *luridus* specimens. Since sweep netting and hand collection were opportunistic, these methods were not standardised between sites. However, the sampling duration of the suction trap and CO_2 baited net remained consistent between sampling sites, and thus offered more useful insights into the species richness and abundance within a region.

The CO_2 baited net collected a diverse group of mosquitoes, producing a total of 16 species. Numerous species were exclusively associated with the baited net, including *Ae*. (*Adm.*) *dentatus*, *Ae*. (*Adm.*) *eritreae*, *Ae*. (*Adm.*) *subdentatus*, *Ae*. (*Neo.*) *luteolateralis*, *Ae*. (*Neo.*) *unidentatus*, *Cx*. (*Cux.*) *simpsoni*

and *Ma*. (*Mnd*.) *africana*. The CO_2 baited net was also responsible for the large number of species sampled at four localities (Bloemfontein semi-pristine habitat 3, Bloemfontein smallholding 2, Bethlehem and Memel). However, the suction trap was only employed at six sampling sites, where it collected relatively few mosquitoes. These species included *Cx*. (*Cux*.) *univittatus* (n = 7), *Cx*. (*Cux*.) *theileri* (n = 6), *Ae*. (*Cat*.) *mixtus* (n = 2), *An*. (*Cel*.) *cydippis* / *squamosus* (n = 2) and *Cx*. (*Cux*.) *pipiens* / *quinquefasciatus* (n = 1), where the suction trap was the only source of *Ae*. (*Cat*.) *mixtus* specimens.

3.3 SPECIES RICHNESS PER SAMPLING LOCALITY

Several patterns of species richness and abundance were observed across the various sampling sites (Table 3.2, Fig. 3.2). Some of the most abundantly sampled species across sampling sites were *Ae*. (*Adm*.) *dentatus* (27.95%), *Ae*. (*Och*.) *juppi* (18.98%), *Cx*. (*Cux*.) *theileri* (14.81%), *Cx*. (*Cux*.) *univittatus* (13.14%) and *Cx*. (*Cux*.) *pipiens* (9.7%). The localities with the greatest species richness included the Bloemfontein semi-pristine habitat 3 (S = 13), Bloemfontein urban habitat 1 (S = 7), Bloemfontein smallholding 2 (S = 6), Bethlehem (S = 6) and Memel (S = 6) sites. However, the high species richness at the Bloemfontein urban habitat 1 site was the product of continuous sampling efforts throughout the sampling period, while the remaining four sites were surveyed in a single evening. The relatively high species richness at four of the five high-richness localities was collected by the standardised CO_2 baited net, which offered more comparable results. These sites represented three habitat types, consisting of smallholdings, semi-urban and semi-pristine sites. Nonetheless, the species richness was generally evenly distributed across the various habitat classifications, except for the farm and rural habitats, which yielded very few species.

Two species were consistently sampled within a specific habitat type, including *Ae*. (*Stg*.) *aegypti* in urban sites and *Ae*. (*Neo*.) *mcintoshi* in semi-pristine habitats. However, several other species were sampled in a wide range of habitat types (*Ae*. *dentatus*, *Ae*. *juppi*, *Cx*. *theileri*, *Cx*. *univittatus*, *Cx*. *pipiens*, *Cx*. *quinquefasciatus*), which may be indicative of their adaptability to novel environments.

Several localities also produced a substantially greater abundance of mosquitoes, including the Bloemfontein semi-pristine habitat 2 (n = 2,953), Bethlehem (n = 463), Bloemfontein smallholding 2 (n = 319) and Hoopstad (n = 187) sites. Here, two high-abundance sites also produced a relatively high species richness (Bethlehem and Bloemfontein smallholding 2). Numerous species were sampled at many localities, including *Cx*. (*Cux*.) *univittatus*, *Cx*. (*Cux*.) *theileri*, *Ae*. (*Och*.) *juppi*, *Cx*. (*Cux*.) *quinquefasciatus* and *Cx*. (*Cux*.) *pipiens*, which were present at six or more sites. Conversely, eight species were only sampled at a single locality, including *Ae*. (*Stg*.) *contiguus*, *Ae*. (*Adm*.) *eritreae*, *Ae*. (*Neo*.) *luridus*, *Ae*. (*Neo*.) *luteolateralis*, *Ae*. (*Cat*.) *mixtus*, *Cx*. (*Mai*.) *salisburiensis*, *Cx*. (*Cux*.) *simpsoni* and *Ma*. (*Mnd*.) *africana*. Here, all species except *Ae*. (*Adm*.) *eritreae* and *Ae*. (*Neo*.) *luridus* consisted of singletons and doubletons.

Table 3.2. Species abundance per sampling locality.

SAMPLING LOCATION	SAMPLED SPECIES	SPECIES ABUNDANCE
Bankfontein - Farm	*Ae. fryeri*	n = 1
Bethlehem - Smallholding	*Ae. dentatus*	n = 260
	Ae. juppi	n = 1
	Ae. subdentatus	n = 2
	Ae. unidentatus	n = 2
	Cx. pipiens / quinquefasciatus	n = 4
	Cx. univittatus	n = 18
Bethulie - Urban	*Ae. contiguus*	n = 1
	Ae. juppi	n = 1
	Cx. salisburiensis	n = 1
	Cx. univittatus	n = 4
Bloemfontein Semi-Pristine Habitat 2 - Semi-pristine	*Ae. caballus*	n = 9
	Ae. juppi	n = 172
	Ae. luridus	n = 3
	Ae. mcintoshi	n = 35
	Cx. univittatus	n = 1
Bloemfontein Semi-Pristine Habitat 3 - Semi-pristine	*Ae. dentatus*	n = 3
	Ae. eritreae	n = 12
	Ae. juppi	n = 1
	Ae. luteolateralis	n = 1
	Ae. mcintoshi	n = 2
	Ae. subdentatus	n = 2
	Ae. unidentatus	n = 2
	An. cydippis / squamosus	n = 1
	Cx. pipiens	n = 9
	Cx. pipiens / quinquefasciatus	n = 4
	Cx. theileri	n = 14
	Cx. univittatus	n = 5
	Ma. africana	n = 1
Bloemfontein Smallholding 1 - Smallholding	*Cx. pipiens*	n = 2
	Cx. theileri	n = 14
	Cx. univittatus	n = 1
	Cs. longiareolata	n = 8
Bloemfontein Smallholding 2 - Smallholding	*Ae. juppi*	n = 1
	Cx. pipiens	n = 74
	Cx. pipiens / quinquefasciatus	n = 31
	Cx. theileri	n = 99
	Cx. univittatus	n = 64
	Cs. longiareolata	n = 2
Bloemfontein Urban Habitat 1 - Urban	*Ae. aegypti*	n = 3
	Ae. caballus	n = 4
	Ae. fryeri	n = 1
	Ae. juppi	n = 3
	Cx. quinquefasciatus	n = 2
	Cx. univittatus	n = 1
	Cs. longiareolata	n = 3
Bloemfontein Urban Habitat 2 - Urban	*Ae. caballus*	n = 14
	Cx. quinquefasciatus	n = 2
	Cs. longiareolata	n = 1
Bloemfontein Urban Habitat 3 - Urban	*Ae. caballus*	n = 1
	Cx. pipiens	n = 1
	Cx. pipiens / quinquefasciatus	n = 1
	Cx. quinquefasciatus	n = 7
	Cx. univittatus	n = 1

SAMPLING LOCATION	SAMPLED SPECIES	SPECIES ABUNDANCE
Boshof - Urban	*Ae. aegypti*	n = 5
	Cx. quinquefasciatus	n = 3
	Cx. simpsoni	n = 1
	Cx. theileri	n = 1
	Cs. longiareolata	n = 1
Bothaville - Urban	*Ae. aegypti*	n = 2
	Cx. pipiens / quinquefasciatus	n = 1
	Cx. theileri	n = 1
Gariep Dam Nature Reserve Location 2 - Pristine	*Cx. pipiens*	n = 5
	Cx. pipiens / quinquefasciatus	n = 1
	Cx. theileri	n = 1
	Cx. univittatus	n = 2
Hoopstad - Urban	*Cx. theileri*	n = 2
	Cx. univittatus	n = 16
Memel - Semi-urban	*Ae. dentatus*	n = 4
	Ae. juppi	n = 1
	Cx. pipiens	n = 2
	Cx. quinquefasciatus	n = 2
	Cx. theileri	n = 3
	Cx. univittatus	n = 9
Oranjeville - Semi-urban	*Cx. pipiens / quinquefasciatus*	n = 2
	Cx. quinquefasciatus	n = 5
Rosendal - Semi-urban	*An. cydippis / squamosus*	n = 2
	Cx. theileri	n = 4
	Cx. univittatus	n = 1
Rural Road Outside Bothaville - Rural	*Ae. juppi*	n = 1
Urban Road in Hoopstad - Urban	*Ae. aegypti*	n = 1
Ventersburg - Rural	*Ae. juppi*	n = 1
Willem Pretorius Game Reserve - Pristine	*Ae. dentatus*	n = 1
	Ae. mixtus	n = 2
	Cx. quinquefasciatus	n = 1
	Cx. theileri	n = 3
	Cx. univittatus	n = 3

Bankfontein
Aedes fryeri
100%
Bethlehem
Aedes dentatus
Aedes juppi
Culex univittatus
Culex pipiens / quinquefasciatus
Aedes unidentatus
Aedes subdentatus
91%
6%
0%
1%
Bethulie
Aedes juppi
Culex univittatus
Aedes contiguus
Culex salisburiensis
15%
57%
14%
14%
Bloemfontein Semi-Pristine Habitat 2
Aedes juppi
Culex univittatus
Aedes mcintoshi
Aedes caballus
Aedes luridus
78%
16%
4%
1%
1%
Bloemfontein Semi-Pristine Habitat 3
Aedes dentatus
Aedes juppi
Culex theileri
Culex univittatus
Culex pipiens
Culex pipiens / quinquefasciatus
Aedes mcintoshi
Aedes eritreae
Aedes unidentatus
Aedes subdentatus
Anopheles cydippis / squamosus
Aedes luteolateralis
Mansonia africana
5%
2%
25%
9%
16%
7%
3%
3%
3%
2%
2%
2%
Bloemfontein Smallholding 1
Culex theileri
Culex univittatus
Culex pipiens
Culiseta longiareolata
56%
4%
8%
32%
Bloemfontein Smallholding 2
Aedes juppi
Culex theileri
Culex univittatus
Culex pipiens
Culex pipiens / quinquefasciatus
Culiseta longiareolata
37%
24%
27%
11%
1%
0%
Bloemfontein Urban Habitat 1
Aedes juppi
Culex univittatus
Aedes caballus
Culex quinquefasciatus
Culiseta longiareolata
Aedes aegypti
Aedes luridus
19%
6%
25%
12%
19%
19%
Bloemfontein Urban Habitat 2
Aedes caballus
Culex quinquefasciatus
Culiseta longiareolata
82%
12%
6%
Bloemfontein Urban Habitat 3
Culex univittatus
Culex pipiens
Culex pipiens / quinquefasciatus
Aedes caballus
Culex quinquefasciatus
9%
9%
9%
9%
64%
Boshof
Culex theileri
Culex quinquefasciatus
Culiseta longiareolata
Aedes aegypti
Culex simpsoni
9%
27%
9%
46%
9%
Bothaville
Culex theileri
Culex pipiens / quinquefasciatus
Aedes aegypti
25%
25%
50%

Fig. 3.2. Species richness and relative abundance per sampling site in the Free State Province. The Bloemfontein semi-pristine habitat 1 site was not included since the sampled specimen was too damaged to be identified.

3.4 BIOGEOGRAPHY

The species distribution maps (Fig. 3.3 - 3.5) supported the seemingly ubiquitous occurrence of numerous species within the Free State Province, including *Ae*. (*Och.*) *juppi*, *Cx.* (*Cux.*) *quinquefasciatus*, *Cx*. (*Cux*.) *theileri* and *Cx*. (*Cux*.) *univittatus*. A few additional species, namely *Ae*. (*Adm.*) *dentatus*, *Ae*. (*Neo.*) *mcintoshi* and *Ae*. (*Neo.*) *unidentatus* were also widespread, but did not occur within the northern regions of the province.

Multiple other species were clustered around the central to south-western regions of the Free State, including *Ae*. (*Stg.*) *aegypti*, *Ae*. (*Och.*) *caballus*, *Ae*. (*Coe.*) *fryeri*, *Ae*. (*Neo.*) *luridus*, *An*. (*Cel.*) *cydippis* / *squamosus*, *Cs*. (*All.*) *longiareolata*, *Cx*. (*Mai.*) *salisburiensis* and *Cx*. (*Cux.*) *pipiens*. *Culex* (*Cux.*) *pipiens* was largely absent from the north-eastern lobe of the province, except for a few sampled specimens at the eastern border.

Several other taxa either had a restricted range or were infrequently sampled, including a cluster of species within the central Free State (*Ae*. *eritreae*, *Ae*. *luteolateralis*, *Ae*. *mixtus*, *Ae*. *subdentatus* and *Ma*. *africana*) and single species located at the southern (*Ae*. *contiguus*) and western (*Cx*. *simpsoni*) borders of the province. Since numerous species were only sampled at a single locality or underrepresented within the literature, the full extent of their distribution could not be determined.

Several of these species were likely sampled in the Free State Province for the first time, since no historical records documenting the presence of *Ae*. (*Adm.*) *eritreae*, *Ae*. (*Adm.*) *subdentatus*, *Ae*. (*Coe.*) *fryeri*, *Ae*. (*Stg.*) *contiguus*, *Cx*. (*Cux.*) *simpsoni* and *Ma*. (*Mnd.*) *africana* in the province could be located (Table 3.3).

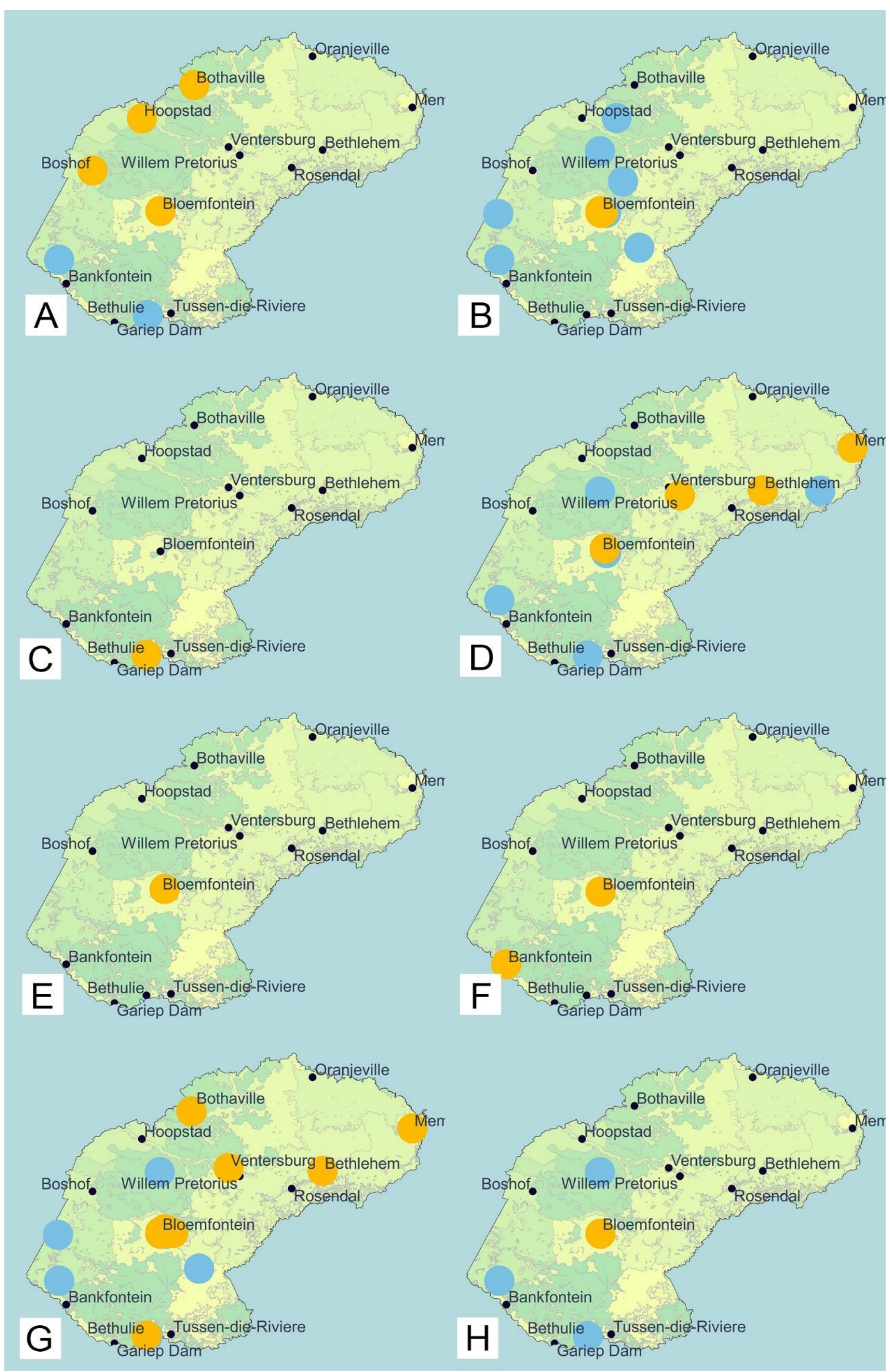


Fig. 3.3. Distribution of sampled *Aedes* species in the Free State Province: a) *Ae. aegypti*; b) *Ae. caballus*; c) *Ae. contiguus*; d) *Ae. dentatus*; e) *Ae. eritreae*; f) *Ae. fryeri*; g) *Ae. juppi*; h) *Ae. luridus*. Data points consist of sampled (orange) and historical (blue) data.

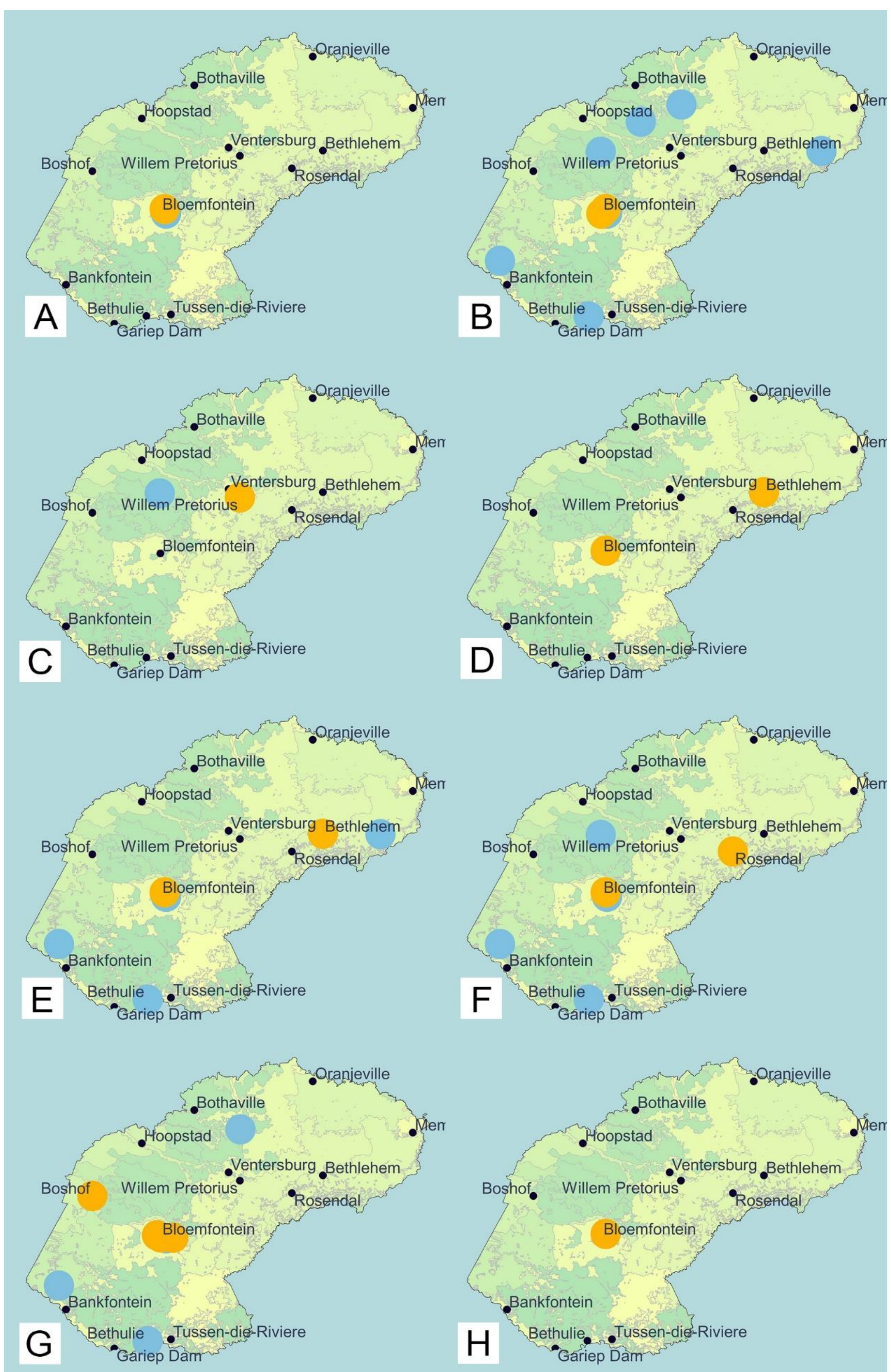


Fig. 3.4. Distribution of sampled *Aedes*, *Anopheles*, *Culiseta* and *Mansonia* species in the Free State Province: a) *Ae. luteolateralis*; b) *Ae. mcintoshi*; c) *Ae. mixtus*; d) *Ae. subdentatus*; e) *Ae. unidentatus*; f) *An. cydippis / squamosus*; g) *Cs. longiareolata*; h) *Ma. africana*. Data points consist of sampled (orange) and historical (blue) data.

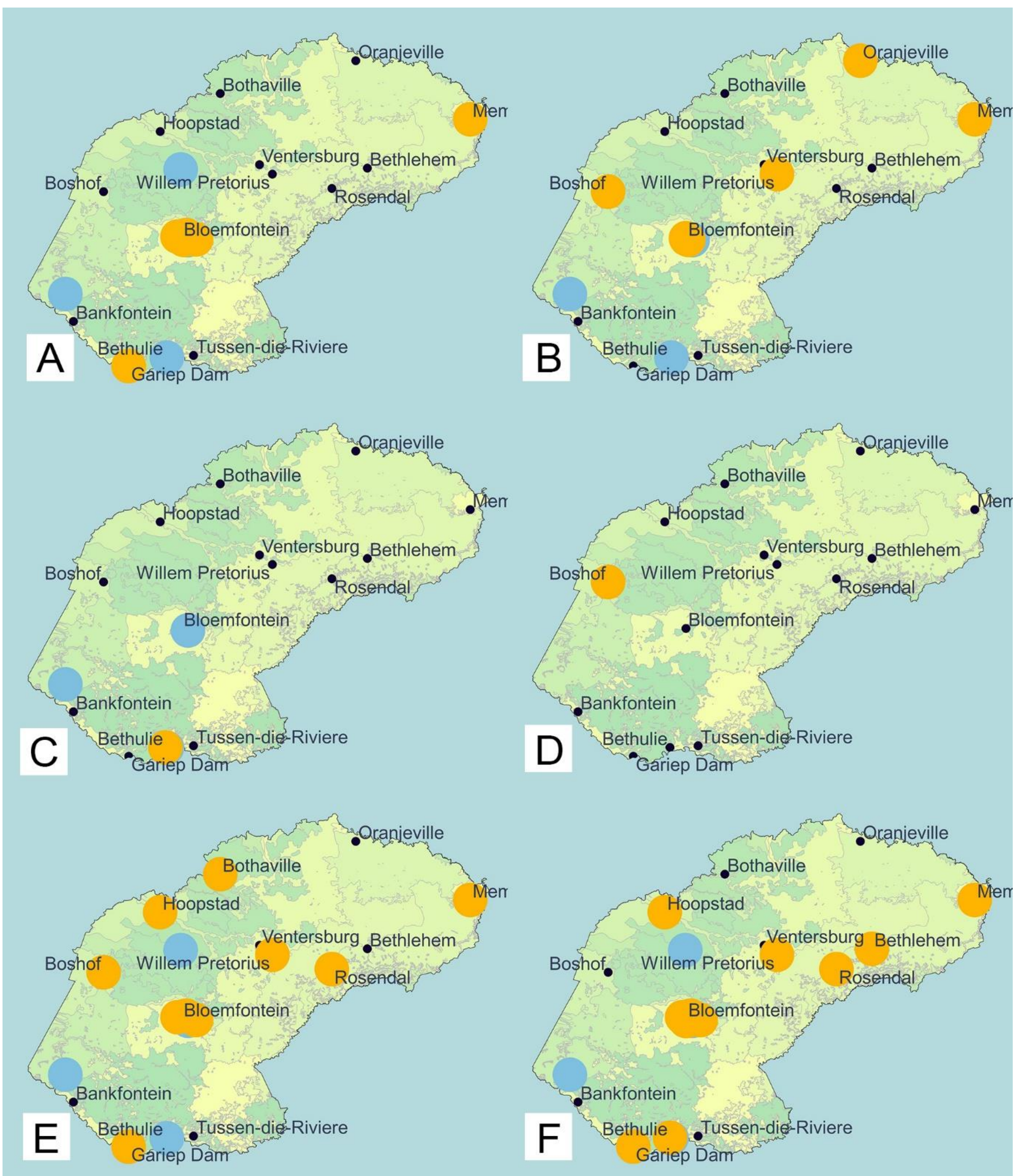


Fig. 3.5. Distribution of sampled *Culex* species in the Free State Province: a) *Cx. pipiens*; b) *Cx. quinquefasciatus*; c) *Cx. salisburiensis*; d) *Cx. simpsoni*; e) *Cx. theileri*; f) *Cx. univittatus*. Data points consist of sampled (orange) and historical (blue) data.

Table 3.3. Sampled species and their documented distribution in the Free State Province.

SPECIES	DISTRIBUTION IN THE FREE STATE	REFERENCES
Ae.* (*Aedimorphus*) *dentatus	Bethulie, Bloemfontein, Bultfontein, Harrismith, Luckhoff	McIntosh 1975, Jupp *et al.* 1980, Jupp & Kemp 1998, Van der Linde *et al.* 1982
Ae.* (*Aedimorphus*) *eritreae	No previous records in the Free State	
Ae.* (*Aedimorphus*) *subdentatus	No previous records in the Free State	
Ae.* (*Catageiomyia*) *mixtus	Bultfontein[1], Vaal River	Bedford 1928 in Muspratt 1955, Jupp & Kemp 1998
Ae.* (*Coetzeemyia*) *fryeri	No previous records in the Free State	
Ae.* (*Neomelaniconion*) *luridus	Bethulie, Bultfontein, Luckhoff	McIntosh 1971, Jupp *et al.* 1980, Jupp & Kemp 1998
Ae.* (*Neomelaniconion*) *luteolateralis	Bloemfontein	Van der Linde *et al.* 1982
Ae.* (*Neomelaniconion*) *mcintoshi	Bethulie, Bloemfontein, Bultfontein, Harrismith, Kroonstad, Luckhoff, Odendaalsrus	Edwards 1941 (*Ae. lineatopennis*), McIntosh 1971 (*Ae. lineatopennis*), Huang 1985, Jupp *et al.* 1980 (*Ae. lineatopennis*), Van der Linde *et al.* 1982 (*Ae. lineatopennis*), Jupp & Kemp 1998
Ae.* (*Neomelaniconion*) *unidentatus	Bethulie, Bloemfontein, Harrismith, Luckhoff, Vaal River	McIntosh 1971, Jupp *et al.* 1980, Van der Linde *et al.* 1982
Ae.* (*Ochlerotatus*) *caballus	Bethulie, Bloemfontein, Brandfort[2], Bultfontein, Dewetsdorp, Jacobsdal, Luckhoff, Vaal River[2], Wesselsbron	Edwards 1941, C.S.I.R. 1954 in Muspratt 1955, Muspratt 1955, McIntosh 1973, McIntosh *et al.* 1980, Jupp *et al.* 1980, Van der Linde *et al.* 1982, Jupp & Kemp 1998, Burt *et al.* 2002
Ae.* (*Ochlerotatus*) *juppi	Bethulie, Bloemfontein, Bultfontein, Dewetsdorp, Jacobsdal, Luckhoff, Vaal River	McIntosh 1973, Jupp *et al.* 1980, McIntosh *et al.* 1980, Van der Linde *et al.* 1982, Jupp & Kemp 1998
Ae.* (*Stegomyia*) *aegypti	Bethulie, Luckhoff	Jupp *et al.* 1980
Ae.* (*Stegomyia*) *contiguus	No previous records in the Free State	
An.* (*Cellia*) *cydippis* / *squamosus	Bethulie[3], Bloemfontein[3], Bultfontein[3], Luckhoff[3]	Jupp *et al.* 1980, Van der Linde *et al.* 1982, Jupp & Kemp 1998
Cx.* (*Culex*) *pipiens	Bethulie, Bloemfontein, Bultfontein, Luckhoff	Jupp 1978, Jupp *et al.* 1980, Van der Linde *et al.* 1982, Jupp & Kemp 1998
Cx.* (*Culex*) *quinquefasciatus	Bethulie, Bloemfontein, Luckhoff	Jupp 1978, Jupp *et al.* 1980, Van der Linde *et al.* 1982
Cx.* (*Culex*) *simpsoni	No previous records in the Free State	
Cx.* (*Culex*) *theileri	Bethulie, Bloemfontein, Bultfontein, Luckhoff, Vaal River	Edwards 1941, Jupp 1978, Jupp *et al.* 1980, Van der Linde *et al.* 1982, Jupp & Kemp 1998
Cx.* (*Culex*) *univittatus	Bethulie, Bloemfontein, Bultfontein, Luckhoff, Vaal River, Welkom	Edwards 1941, Jupp 1978, Jupp *et al.* 1980, Van der Linde *et al.* 1982, Jupp & Kemp 1998, Burt *et al.* 2002
Cx.* (*Maillotia*) *salisburiensis	Bethulie, Bloemfontein, Luckhoff	Jupp *et al.* 1980, Van der Linde *et al.* 1982
Cs.* (*Allotheobaldia*) *longiareolata	Bethulie, Bloemfontein, Kroonstad, Luckhoff	Edwards 1941, Jupp 1978, Jupp *et al.* 1980, Van der Linde *et al.* 1982
Ma.* (*Mansonioides*) *africana	No previous records in the Free State	

1. Ae. *mixtus* / *microstictus*
2. *Ae. caballus* s.l.
3. Does not include *An. cydippis*

3.5 INTRASPECIFIC MORPHOLOGICAL VARIATION

Instances of morphological variation were noted for each species, including features of the habitus, the presence of setae and the patterning on the integument, head, thorax, legs and abdomen. Many cases of variation occurred infrequently or consisted of unique combinations of characters, rather than co-occurring morphological features. Several instances of morphological variation was observed within *Aedes*, including *Ae*. (*Adm*.) *dentatus*, which periodically presented with dark lateral markings on the head (Fig. 3.6 a, b) and a single *Ae*. (*Neo*.) cf. *mcintoshi* specimen with unusually light coloured sterna (AED20.405; Fig. 3.6 c, d). Furthermore, a relatively large *Ae*. (*Och*.) *caballus* male uncharacteristically presented with lower mesanepimeral setae (LMS) (AED18.4; Fig. 3.6 e, f), thus contradicting the description of McIntosh (1973).

Similarly, a significant degree of morphological variation was observed within numerous *Culex* species. A single *Cx*. (*Cux*.) *pipiens* specimens was relatively robust and presented with dark colouration (CLX12.10), while another displayed asymmetry regarding the number of lower mesanepimeral setae (CLX2.90; Fig. 3.6 g, h). The closely related *Cx*. (*Cux*.) *quinquefasciatus* consisted of one relatively small specimen (CLX13.5) and one specimen with reduced abdominal patterning (CLX4.2.19; Fig. 3.6 i, j). Three *Cx*. (*Cux*.) *theileri* specimens also displayed unique characteristics, including individuals with light (CLX1.6) or dark (CLX1.11) coloured integuments and one specimen with additional spots on tergites 4, 5 and 6 (CLX2.21; Fig. 3.6 k, l), which has been documented by Edwards (1941). Finally, *Cx*. (*Cux*.) *univittatus* displayed a relatively large degree of morphological variation, where only a few characteristics were shared between specimens. Several individuals were relatively small (CLX1.16, CLX2.172, CLX2.243, CLX7.4) and two of these specimens also possessed large post-spiracular scale patches (CLX2.172, CLX2.243; Fig. 3.6 m, n). Since these specimens were well-preserved, the variation in the scale patches was assumed to be innate characteristics rather than a by-product of specimen damage. Variation was also observed in the leg patterning of *Cx*. (*Cux*.) *univittatus*, where two specimens had a prominent pale stripe on the midfemur (CLX12.3, CLX2.243; Fig. 3.6 o, p), while one darkly coloured specimen had a relatively short dorsal stripe on the hind femur (CLX2.69; Fig. 3.6 q, r). The remaining specimens had prominent leg colouration (CLX2.256) or were delicate and lightly coloured (CLX7.4). Within *Cx*. (*Cux*.) *univittatus*, Edwards (1941) noted several examples of morphological variation, including differences in the abdominal patterning and length of the hind femur's stripe.

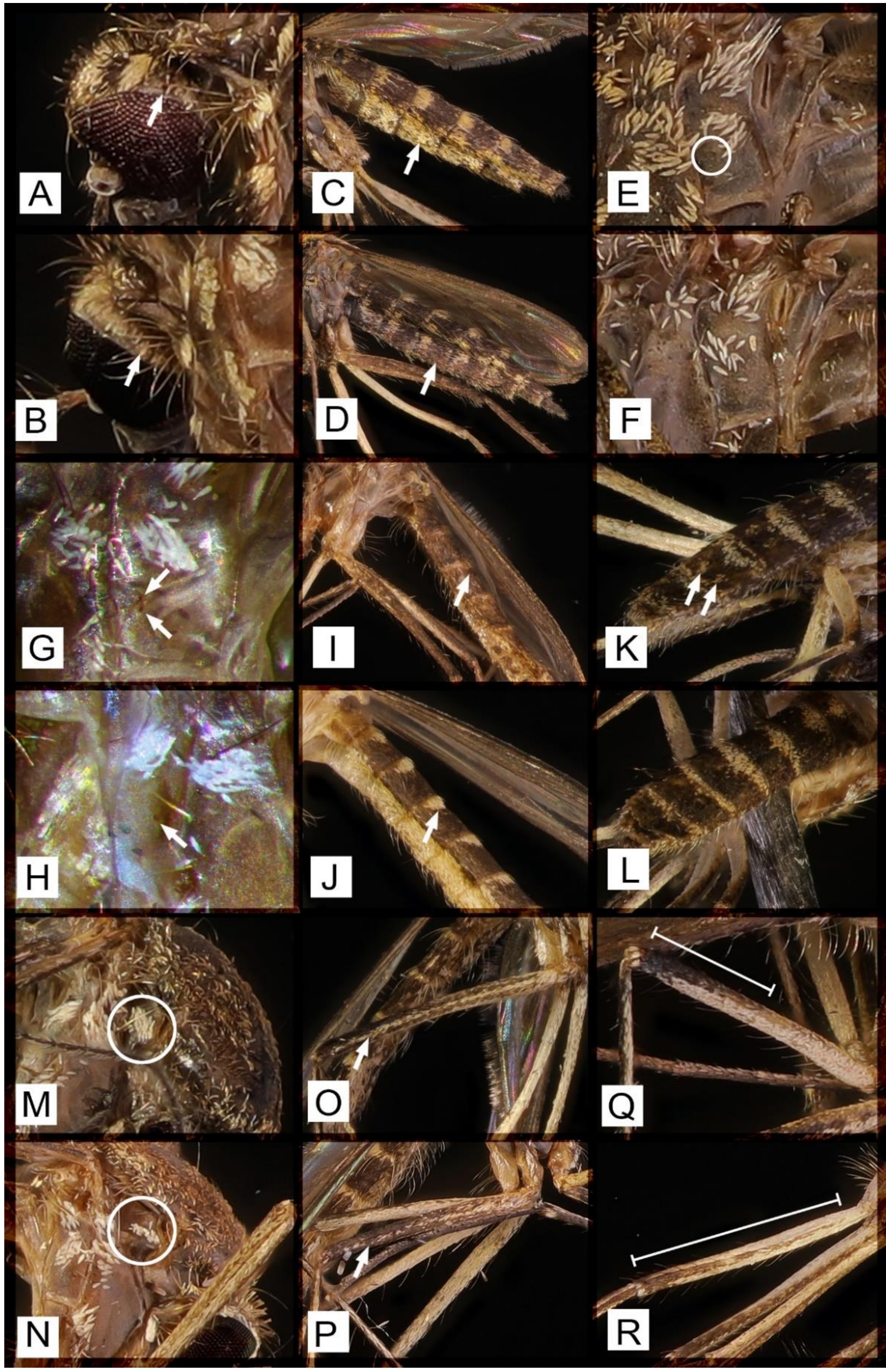


Fig. 3.6. The morphological variation observed in sampled specimens: *Ae.* (*Adm.*) *dentatus* with (a) and without (b) dark lateral head markings; *Ae.* (*Neo.*) *mcintoshi* with light (c) and typical (d) coloured sterna; *Ae.* (*Och.*) *caballus* male with (e) and without (f) LMS; *Cx.* (*Cux.*) *pipiens* with an asymmetrical number of LMS (g, h); *Cx.* (*Cux.*) *quinquefasciatus* with reduced (i) and typical (j) tergal markings; *Cx.* (*Cux.*) *theileri* with atypical (k) and typical (l) tergal markings, *Cx.* (*Cux.*) *univittatus* with large (m) and elongate (n) postspiracular scale patches; *Cx.* (*Cux.*) *univittatus* with prominent (o) and typical (p) midfemur markings; *Cx.* (*Cux.*) *univittatus* with a proportionally short (q) and typical (r) dorsal hind femur stripe.

3.6 DNA SEQUENCE IDENTIFICATION

Of the 88 selected sampled specimens, 62 were successfully sequenced for at least one of the three target DNA regions, including 58 for the COI region, 22 for the ITS2 region and 8 for the 28S region (Table 3.4). Since most sampled species were also represented within the GenBank database (Benson *et al.* 2012), generated sequences from each specimen were entered as a query within the BLAST search function.

When considering the species represented within the GenBank database, the highest scoring BLAST results matched 91% of the sampled specimens' morphologically assigned species identities. Here, all sampled *Cx.* (*Cux.*) *quinquefasciatus* specimens (CLX4.2.19, CLX13.5, CLX18.2, CLX19.2, CLX21.5) yielded similar sequence identities to both *Cx.* (*Cux.*) *quinquefasciatus* and *Cx.* (*Cux.*) *pipiens* specimens in the GenBank database. On the other hand, all sampled *Cx.* (*Cux.*) *pipiens* specimens only produced conspecific BLAST matches. Since these two closely related species are morphologically similar, individual database records may not always reflect to correct species identity and a consensus of matches need to be taken into account.

The morphologically assigned species identities of five sampled specimens did not match the top BLAST results when arranged by percent identity, despite these species being represented within the database. This included two *Ae.* (*Neo.*) *unidentatus* specimens (AED8.30, AED22.18), two *Ae.* (*Och.*) *juppi* specimens (AED9.1, AED12.1) and one morphologically divergent *Ae.* (*Neo.*) cf. *mcintoshi* specimen (AED20.405). The top five identity matches for the sampled *Ae.* (*Neo.*) *unidentatus* specimens consisted of intermixed database entries of *Ae.* (*Neo.*) *unidentatus* and *Ae.* (*Neo.*) *mcintoshi* sequences. Additionally, the two sampled *Ae.* (*Och.*) *juppi* specimens were matched to a closely related species, *Ae.* (*Och.*) *caballus*, while the sampled *Ae.* (*Neo.*) cf. *mcintoshi* specimen had a high identity match to *Ae.* (*Neo.*) *unidentatus* (99%). Therefore, the identities of these sampled *Ae.* (*Neo.*) *unidentatus* and *Ae.* (*Och.*) *juppi* specimens were reconfirmed with morphological identifications. However, the *Ae.* (*Neo.*) cf. *mcintoshi* specimen lost discernible features during sequencing and its identity could therefore not be reaffirmed. Nonetheless, this specimen was well-preserved during sampling and all the necessary morphological characteristics were retained during the initial round of identifications. The *Ae.* (*Neo.*) cf. *mcintoshi* sequence query also produced a close match with *Ae.* (*Neo.*) *mcintoshi* when results were arranged by the E-value, rather than percent identity.

Furthermore, eight of the sampled species were not represented within the GenBank database. No comparable sequences were available for sampled individuals of *Ae.* (*Cat.*) *mixtus* (AED4.2.2), *Ae.* (*Stg.*) *contiguus* (AED18.14), *Ae.* (*Coe.*) *fryeri* (AED18.12), *Ae.* (*Neo.*) *luridus* (AED20.1720), *Ae.* (*Neo.*) *luteolateralis* (AED22.28) and *Cx.* (*Mai.*) *salisburiensis* (CLX9.2). Nonetheless, all sampled *Aedes* specimens matched with database specimens from their respective subgenera, except for the sampled *Ae.* (*Coe.*) *fryeri*, where no other database sequences were available for the monotypic subgenus *Coetzeemyia*.

The sampled *Ae.* (*Neo.*) *luridus* specimen shared a significant sequence identity with *Ae.* (*Neo.*) *mcintoshi* (99%) on the database. Therefore, the specimen was re-identified morphologically and its assigned species as *Ae.* (*Neo.*) *luridus* was reconfirmed. Within *Culex*, the sampled *Cx.* (*Mai.*) *salisburiensis* specimen matched with another member of the subgenus *Maillotia* on the database when arranged by the E-value results. Also, one sampled *Cx.* (*Cux.*) *univittatus* specimen (CLX2.243) was only successfully sequenced for the ITS2 region, a region that was not represented within the GenBank database for this species. Nevertheless, this specimen's ITS2 BLAST results were analogous to the matches of other sampled *Cx.* (*Cux.*) *univittatus* specimens.

The top identity values within the BLAST results frequently ranged from 95-100%, potentially revealing taxonomic challenges or a large degree of genetic variation. Three sampled specimens were still correlated with their assigned species despite lower identity values (< 95%). This included *Ae.* (*Och.*) *juppi* (AED20.107, 93.46%) and two *An.* (*Cel.*) *cydippis* / *squamosus* specimens (ANO7.1, 94.03%; ANO22.1, 87.29%). Here, sampled *An.* (*Cel.*) *cydippis* / *squamosus* specimens may have belonged to one of two species, while only *An.* (*Cel.*) *squamosus* sequences were available on the database for comparison. Therefore, low identity values would have been expected if sampled specimens were members of *An.* (*Cel.*) *cydippis*. However, these three generated sequences also contained a relatively large number of undetermined nucleotides, which may have contributed to the low sequence identities.

Table 3.4. BLAST percentage identity (PI) and E value (E) results for successfully sequenced specimens.

SAMPLED SPECIMENS		BLAST RESULTS								
Species	**Specimen**	**COI**			**ITS2**			**28S**		
Ae.* (*Stg.*) *aegypti	AED14.1.1	*Ae.* (*Stg.*) *aegypti*	100%	PI						
Ae.* (*Stg.*) *aegypti	AED18.11	*Ae.* (*Stg.*) *aegypti*	100%	PI						
Ae.* (*Och.*) *caballus	AED18.4	*Ae.* (*Och.*) *caballus*	97%	PI	*Ae.* (*Och.*) *caballus*	95%	PI			
Ae.* (*Och.*) *caballus	AED19.3	*Ae.* (*Och.*) *caballus*	97%	PI	*Ae.* (*Och.*) *caballus*	96%	PI			
Ae.* (*Och.*) *caballus	AED19.11	*Ae.* (*Och.*) *caballus*	97%	PI	*Ae.* (*Och.*) *caballus*	96%	PI			
Ae.* (*Och.*) *caballus	AED20.2707	*Ae.* (*Och.*) *caballus*	97%	PI	*Ae.* (*Och.*) *impiger*	95%	PI			
Ae.* (*Och.*) *caballus	AED21.1	*Ae.* (*Och.*) *caballus*	97%	PI	*Ae.* (*Och.*) *diantaeus*	95%	PI			
Ae.* (*Och.*) *caballus	AED18.14	*Ae.* (*Och.*) *caballus*	96%	PI						
Ae.* (*Stg.*) *contiguus	AED9.2	*Ae.* (*Stg.*) *ledgeri*	99%	PI						
Ae.* (*Adm.*) *dentatus	AED12.2				*Ae.* (*Adm.*) *pallidostriatus*	100%	PI	*Ae.* (*Adm.*) *vexans*	86%	PI
Ae.* (*Adm.*) *eritreae	AED22.16	*Ae.* (*Adm.*) *eritreae*	96%	PI						
Ae.* (*Coe.*) *fryeri	AED18.12							*Armigeres* (*Arm.*) *subalbatus*	92%	PI
								Ae. (*Adm.*) *vexans*	89%	E
Ae.* (*Och.*) *juppi	AED2.1	*Ae.* (*Och.*) *juppi*	100%	PI				*Ae.* (*Pro.*) *triseriatus*	100%	PI
Ae.* (*Och.*) *juppi	AED9.1	*Ae.* (*Och.*) *caballus*	100%	PI						
Ae.* (*Och.*) *juppi	AED12.1	*Ae.* (*Och.*) *caballus*	100%	PI						
Ae.* (*Och.*) *juppi	AED18.9	*Ae.* (*Och.*) *juppi*	100%	PI	*Ae.* (*Och.*) *caballus*	92%	PI	*Ae.* (*Dah.*) *geniculatus*	98%	
Ae.* (*Och.*) *juppi	AED20.107	*Ae.* (*Och.*) *juppi*	93%	PI						
Ae.* (*Och.*) *juppi	AED20.2704	*Ae.* (*Och.*) *juppi*	99%	PI	*Ae.* (*Och.*) *cataphylla*	95%	PI			
Ae.* (*Neo.*) *luridus	AED20.1720	*Ae.* (*Neo.*) *mcintoshi*	99%	PI	*Ae.* (*Neo.*) *mcintoshi*	94%	PI			
Ae.* (*Neo.*) *luteolateralis	AED22.28	*Ae.* (*Neo.*) *unidentatus*	98%	PI						
Ae.* (*Neo.*) *mcintoshi	AED20.2710	*Ae.* (*Neo.*) *mcintoshi*	97%	PI	*Ae.* (*Neo.*) *mcintoshi*	97%	PI			
Ae.* (*Neo.*) *mcintoshi	AED22.19	*Ae.* (*Neo.*) *mcintoshi*	96%	PI						
Ae.* (*Neo.*) cf. *mcintoshi	AED20.405	*Ae.* (*Neo.*) *unidentatus*	99%	PI	*Ae.* (*Neo.*) *mcintoshi*	98%	PI			
		Ae. (*Neo.*) *mcintoshi*	98%	E	*Ae.* (*Neo.*) *mcintoshi*	98%	PI			
Ae.* (*Cat.*) *mixtus	AED4.2.2	*Ae.* (*Cat.*) *microstictus*	99%	PI						
Ae.* (*Neo.*) *unidentatus	AED8.30	*Ae.* (*Neo.*) *mcintoshi*	100%	PI						
		Ae. (*Neo.*) *unidentatus*	99%	PI						
Ae.* (*Neo.*) *unidentatus	AED22.18	*Ae.* (*Neo.*) *mcintoshi*	96%	PI						
		Ae. (*Neo.*) *unidentatus*	95%	PI						
Cx.* (*Cux.*) *pipiens	CLX2.82	*Cx.* (*Cux.*) *pipiens*	96%							
Cx.* (*Cux.*) *pipiens	CLX2.90	*Cx.* (*Cux.*) *pipiens*	100%	PI						
Cx.* (*Cux.*) *pipiens	CLX2.280	*Cx.* (*Cux.*) *pipiens*	100%	PI	*Cx.* (*Cux.*) *pipiens*	100%	PI			
Cx.* (*Cux.*) *pipiens	CLX5.2.7	*Cx.* (*Cux.*) *pipiens*	99%	PI						
Cx.* (*Cux.*) *pipiens	CLX12.10	*Cx.* (*Cux.*) *pipiens*	98%	PI						
Cx.* (*Cux.*) *pipiens	CLX21.10	*Cx.* (*Cux.*) *pipiens*	99%	PI	*Cx.* (*Cux.*) *pipiens*	95%	PI			
Cx.* (*Cux.*) *pipiens	CLX22.42	*Cx.* (*Cux.*) *pipiens*	98%	PI						
Cx.* (*Cux.*) *quinquefasciatus	CLX4.2.19	*Cx.* (*Cux.*) *quinquefasciatus*	99%	PI						
Cx.* (*Cux.*) *quinquefasciatus	CLX13.5	*Cx.* (*Cux.*) *quinquefasciatus*	96%	PI						

Species	Specimen	COI			ITS2			28S		
Cx.* (*Cux.*) *quinquefasciatus	CLX18.2	*Cx.* (*Cux.*) *quinquefasciatus*	100%	PI						
Cx.* (*Cux.*) *quinquefasciatus	CLX19.2	*Cx.* (*Cux.*) *quinquefasciatus*	100%	PI						
Cx.* (*Cux.*) *quinquefasciatus	CLX21.5	*Cx.* (*Cux.*) *quinquefasciatus*	100%	PI						
Cx.* (*Mai.*) *salisburiensis	CLX9.2	*Cx.* (*Cux.*) *restuans*	91%	PI						
		Cx. (*Mai.*) *hortensis*	91%	E						
Cx.* (*Cux.*) *theileri	CLX1.6	*Cx.* (*Cux.*) *theileri*	100%	PI						
Cx.* (*Cux.*) *theileri	CLX1.11	*Cx.* (*Cux.*) *theileri*	100%	PI						
Cx.* (*Cux.*) *theileri	CLX2.21				*Cx.* (*Cux.*) *theileri*	96%	PI			
Cx.* (*Cux.*) *theileri	CLX2.24	*Cx.* (*Cux.*) *theileri*	99%	PI	*Cx.* (*Cux.*) *theileri*	96%	PI	*Cx.* (*Cux.*) *theileri*	99%	PI
Cx.* (*Cux.*) *theileri	CLX4.2.5	*Cx.* (*Cux.*) *theileri*	98%	PI	*Cx.* (*Cux.*) *theileri*	98%	PI			
Cx.* (*Cux.*) *theileri	CLX15.1	*Cx.* (*Cux.*) *theileri*	100%	PI						
Cx.* (*Cux.*) *univittatus	CLX1.16	*Cx.* (*Cux.*) *univittatus*	100%	PI	*Cx.* (*Cux.*) *pseudovishnui*	86%	PI			
Cx.* (*Cux.*) *univittatus	CLX2.68	*Cx.* (*Cux.*) *univittatus*	100%	PI				*Cx.* (*Cux.*) *annulus*	99%	PI
Cx.* (*Cux.*) *univittatus	CLX2.69	*Cx.* (*Cux.*) *univittatus*	99%	PI	*Cx.* (*Cux.*) *quinquefasciatus*	76%	PI			
Cx.* (*Cux.*) *univittatus	CLX2.172	*Cx.* (*Cux.*) *univittatus*	96%	PI	*Cx.* (*Cux.*) *pseudovishnui*	86%	PI			
Cx.* (*Cux.*) *univittatus	CLX2.243				*Cx.* (*Cux.*) *pseudovishnui*	86%	PI			
Cx.* (*Cux.*) *univittatus	CLX2.256	*Cx.* (*Cux.*) *univittatus*	100%	PI				*Cx.* (*Cux.*) *annulus*	99%	PI
Cx.* (*Cux.*) *univittatus	CLX4.2.1	*Cx.* (*Cux.*) *univittatus*	97%	PI						
Cx.* (*Cux.*) *univittatus	CLX4.2.9	*Cx.* (*Cux.*) *univittatus*	100%	PI	*Cx.* (*Cux.*) *pseudovishnui*	86%	PI	*Cx.* (*Cux.*) *perexiguus*	98%	PI
Cx.* (*Cux.*) *univittatus	CLX7.4	*Cx.* (*Cux.*) *univittatus*	97%	PI						
Cx.* (*Cux.*) *univittatus	CLX8.3	*Cx.* (*Cux.*) *univittatus*	100%	PI						
Cx.* (*Cux.*) *univittatus	CLX9.4	*Cx.* (*Cux.*) *univittatus*	100%	PI						
Cx.* (*Cux.*) *univittatus	CLX12.3	*Cx.* (*Cux.*) *univittatus*	100%	PI						
Cx.* (*Cux.*) *univittatus	CLX12.5	*Cx.* (*Cux.*) *univittatus*	100%	PI						
Cx.* (*Cux.*) *univittatus	CLX18.3	*Cx.* (*Cux.*) *univittatus*	98%	PI						
An.* (*Cel.*) *cydippis* / *squamosus	ANO7.1	*An.* (*Cel.*) *squamosus*	94%	PI						
An.* (*Cel.*) *cydippis* / *squamosus	ANO22.1	*An.* (*Cel.*) *squamosus*	87%	PI						
***An.* (*Cel.*) sp.**	ANO3.1	*An.* (*Cel.*) *squamosus*	92%	PI	*An.* (*Cel.*) *squamosus*	94%	PI			

3.7 PHYLOGENETIC ANALYSES

The phylogenetic investigations of the three target genera (*Aedes*, *Culex* and *Anopheles*) produced a total of 18 trees, which spanned three DNA regions and two phylogenetic methods. The datasets incorporated a total of 94 generated sequences, where the morphological identities of sampled specimens were corroborated by BLAST queries or morphologically reaffirmed when sequence comparisons were unavailable or ambiguous. These identities were substantiated by the phylogenetic placement of sampled individuals, consisting of conspecific groupings or clusters of closely related species.

Within the phylogenetic results, each genus was represented by a total of six trees, which often yielded unique topologies. However, this was largely due to differences in taxon sampling, since limited sequences were available for the ITS2 and 28S DNA regions. These topologies were also dependent on the phylogenetic method, since the maximum likelihood trees achieved a greater topological resolution, yet with weaker support in basal clades. Nonetheless, numerous affiliations remained consistent between analyses despite the topological differences, and thus reflected a shared phylogenetic signal. These consistent associations were examined from a South African perspective, where the interpretations were centred on taxa relevant to the region.

While comparing the haplotypes of South African and non-South African specimens belonging to sampled species, patterns of unique and shared alleles often revealed internal stratification according to their geographic location, which similarly provided insight into the basis for the phylogenetic groupings. Such stratification was however not universal, and several species displayed remarkably homogenous haplotypes.

3.7.1 *AEDES*

The *Aedes* datasets were represented by 25 subgenera, 53 species and 249 sequences across the three DNA regions. Two of the larger subgenera, *Ochlerotatus* and *Stegomyia*, were additionally represented by five and six species groups, respectively. The datasets included 32 species occurring in South Africa, where 39 sequences were generated from 12 sampled species. The datasets incorporated two morphologically atypical specimens, including one *Ae*. (*Neo*.) cf. *mcintoshi* specimen (AED20.405, Fig. 3.6 c) with uncharacteristically light coloured sterna, which nonetheless produced a 98% identity match with another *Ae*. (*Neo*.) *mcintoshi* specimen on the GenBank database. The dataset also included a relatively large and atypical *Ae*. (*Och*.) *caballus* male with LMS (AED18.4, Fig. 3.6 e), which similarly produced a conspecific match within the BLAST results.

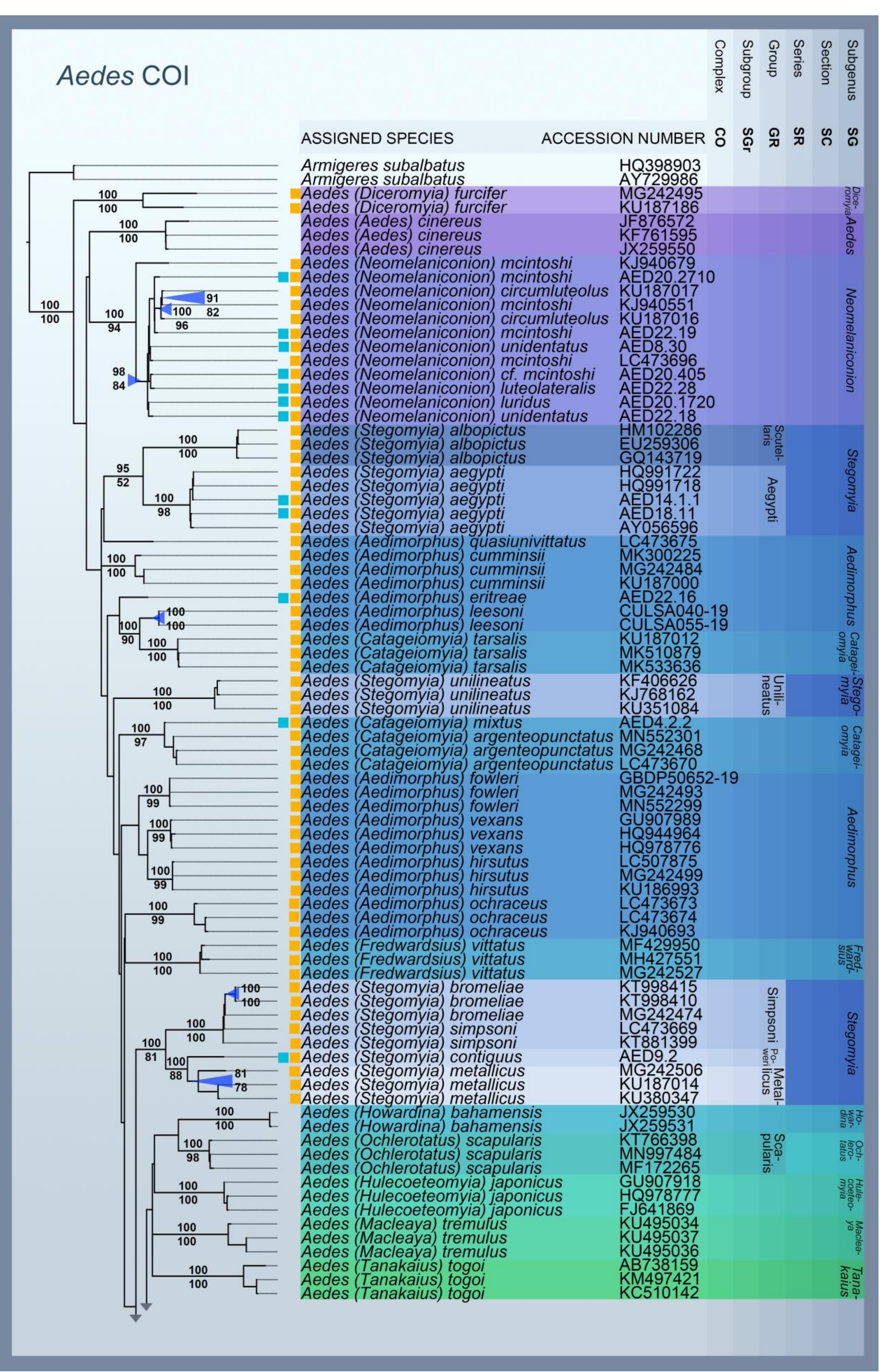
The COI dataset was the largest and represented 22 subgenera and 47 species. The alignment consisted of 657 sites, including 391 conserved, 266 variable and 246 parsimony informative positions. Terminal relationships were moderately to well-supported, while the deeper relationships had a low degree of phylogenetic resolution (Fig. 3.7). Nonetheless, numerous groupings remained consistent between the phylogenetic methods. Several subgenera were represented by a single species with weakly supported or inconsistent affiliations, while many of the well-represented subgenera were recovered as polyphyletic.

The ITS2 region represented the second-largest dataset, with 21 subgenera and 36 species. Here, the alignment consisted of 591 sites with 231 conserved, 328 variable and 283 parsimony informative positions. Both the ITS2 and COI results shared topological features, including the relatively weakly resolved basal relationships and a greater degree of support in terminal clades (Fig. 3.8). Once again, numerous clades and affiliations remained consistent between the phylogenetic methods. Here, many

subgenera were also represented by a single species, while the larger subgenera and a few species consisted of non-monophyletic assemblages. The non-monophyletic species groupings may have been a by-product of misidentified specimens, a reflection of unresolved taxonomic issues or caused by the DNA region's phylogenetic limitations.

Finally, the 28S dataset included seven subgenera and ten species, with sequences spanning the D1 domain. The alignment consisted of 305 sites, with 267 conserved, 38 variable and 32 parsimony informative positions. The analyses included relatively few species, yet still produced a few non-monophyletic subgenera (Fig. 3.9). The support for basal and terminal relationships varied between the phylogenetic methods, where terminal interspecific groupings resulted in polytomies or weakly supported affiliations. The two analyses also differed noticeably in their topologies, likely due to the limited phylogenetic signal associated with the conserved 28S region. Here, the analyses produced two polyphyletic subgenera with inconsistent affiliations.

Numerous findings were supported within multiple analyses, including the polyphyly of *Aedimorphus*, *Ochlerotatus* and *Stegomyia*. *Ochlerotatus* often shared clades with two other subgenera, namely *Downsiomyia* and *Jarnellius*. *Stegomyia*'s Metallicus Group was also consistently associated with the Simpsoni Group. Another subgenus, *Neomelaniconion*, was also reliably affiliated with the subgenus *Aedes*. Lastly, the subgenus *Catageiomyia* was only represented within the COI analyses, where it was also recovered as a polyphyletic assemblage. Since the COI and ITS2 datasets had the greatest taxonomic coverage, these DNA regions recovered the most comparable results.



Aedes COI
ASSIGNED SPECIES
ACCESSION NUMBER
CO
SGr
GR
SR
SC
SG
Complex
Subgroup
Group
Series
Section
Subgenus
Armigeres subalbatus HQ398903
Armigeres subalbatus AY729986
Aedes (Diceromyia) furcifer MG242495
Aedes (Diceromyia) furcifer KU187186
Aedes (Aedes) cinereus JF876572
Aedes (Aedes) cinereus KF761595
Aedes (Aedes) cinereus JX259550
Aedes (Neomelaniconion) mcintoshi KJ940679
Aedes (Neomelaniconion) mcintoshi AED20.2710
Aedes (Neomelaniconion) circumluteolus KU187017
Aedes (Neomelaniconion) mcintoshi KJ940551
Aedes (Neomelaniconion) circumluteolus KU187016
Aedes (Neomelaniconion) mcintoshi AED22.19
Aedes (Neomelaniconion) unidentatus AED8.30
Aedes (Neomelaniconion) mcintoshi LC473696
Aedes (Neomelaniconion) cf. mcintoshi AED20.405
Aedes (Neomelaniconion) luteolateralis AED22.28
Aedes (Neomelaniconion) luridus AED20.1720
Aedes (Neomelaniconion) unidentatus AED22.18
Aedes (Stegomyia) albopictus HM102286
Aedes (Stegomyia) albopictus EU259306
Aedes (Stegomyia) albopictus GQ143719
Aedes (Stegomyia) aegypti HQ991722
Aedes (Stegomyia) aegypti HQ991718
Aedes (Stegomyia) aegypti AED14.1.1
Aedes (Stegomyia) aegypti AED18.11
Aedes (Stegomyia) aegypti AY056596
Aedes (Aedimorphus) quasiunivittatus LC473675
Aedes (Aedimorphus) cumminsii MK300225
Aedes (Aedimorphus) cumminsii MG242484
Aedes (Aedimorphus) cumminsii KU187000
Aedes (Aedimorphus) eritreae AED22.16
Aedes (Aedimorphus) leesoni CULSA040-19
Aedes (Aedimorphus) leesoni CULSA055-19
Aedes (Catageiomyia) tarsalis KU187012
Aedes (Catageiomyia) tarsalis MK510879
Aedes (Catageiomyia) tarsalis MK533636
Aedes (Stegomyia) unilineatus KF406626
Aedes (Stegomyia) unilineatus KJ768162
Aedes (Stegomyia) unilineatus KU351084
Aedes (Catageiomyia) mixtus AED4.2.2
Aedes (Catageiomyia) argenteopunctatus MN552301
Aedes (Catageiomyia) argenteopunctatus MG242468
Aedes (Catageiomyia) argenteopunctatus LC473670
Aedes (Aedimorphus) fowleri GBDP50652-19
Aedes (Aedimorphus) fowleri MG242493
Aedes (Aedimorphus) fowleri MN552299
Aedes (Aedimorphus) vexans GU907989
Aedes (Aedimorphus) vexans HQ944964
Aedes (Aedimorphus) vexans HQ978776
Aedes (Aedimorphus) hirsutus LC507875
Aedes (Aedimorphus) hirsutus MG242499
Aedes (Aedimorphus) hirsutus KU186993
Aedes (Aedimorphus) ochraceus LC473673
Aedes (Aedimorphus) ochraceus LC473674
Aedes (Aedimorphus) ochraceus KJ940693
Aedes (Fredwardsius) vittatus MF429950
Aedes (Fredwardsius) vittatus MH427551
Aedes (Fredwardsius) vittatus MG242527
Aedes (Stegomyia) bromeliae KT998415
Aedes (Stegomyia) bromeliae KT998410
Aedes (Stegomyia) bromeliae MG242474
Aedes (Stegomyia) simpsoni LC473669
Aedes (Stegomyia) simpsoni KT881399
Aedes (Stegomyia) contiguus AED9.2
Aedes (Stegomyia) metallicus MG242506
Aedes (Stegomyia) metallicus KU187014
Aedes (Stegomyia) metallicus KU380347
Aedes (Howardina) bahamensis JX259530
Aedes (Howardina) bahamensis JX259531
Aedes (Ochlerotatus) scapularis KT766398
Aedes (Ochlerotatus) scapularis MN997484
Aedes (Ochlerotatus) scapularis MF172265
Aedes (Hulecoeteomyia) japonicus GU907918
Aedes (Hulecoeteomyia) japonicus HQ978777
Aedes (Hulecoeteomyia) japonicus FJ641869
Aedes (Macleaya) tremulus KU495034
Aedes (Macleaya) tremulus KU495037
Aedes (Macleaya) tremulus KU495036
Aedes (Tanakaius) togoi AB738159
Aedes (Tanakaius) togoi KM497421
Aedes (Tanakaius) togoi KC510142
Diceromyia
Aedes
Neomelaniconion
Scutellaris
Aegypti
Stegomyia
Aedimorphus
Catageiomyia
Unilineatus
Stegomyia
Catageiomyia
Aedimorphus
Fredwardsius
Simpsoni
Poweri
Metallicus
Stegomyia
Howardina
Scapularis
Ochlerotatus
Hulecoeteomyia
Macleaya
Tanakaius

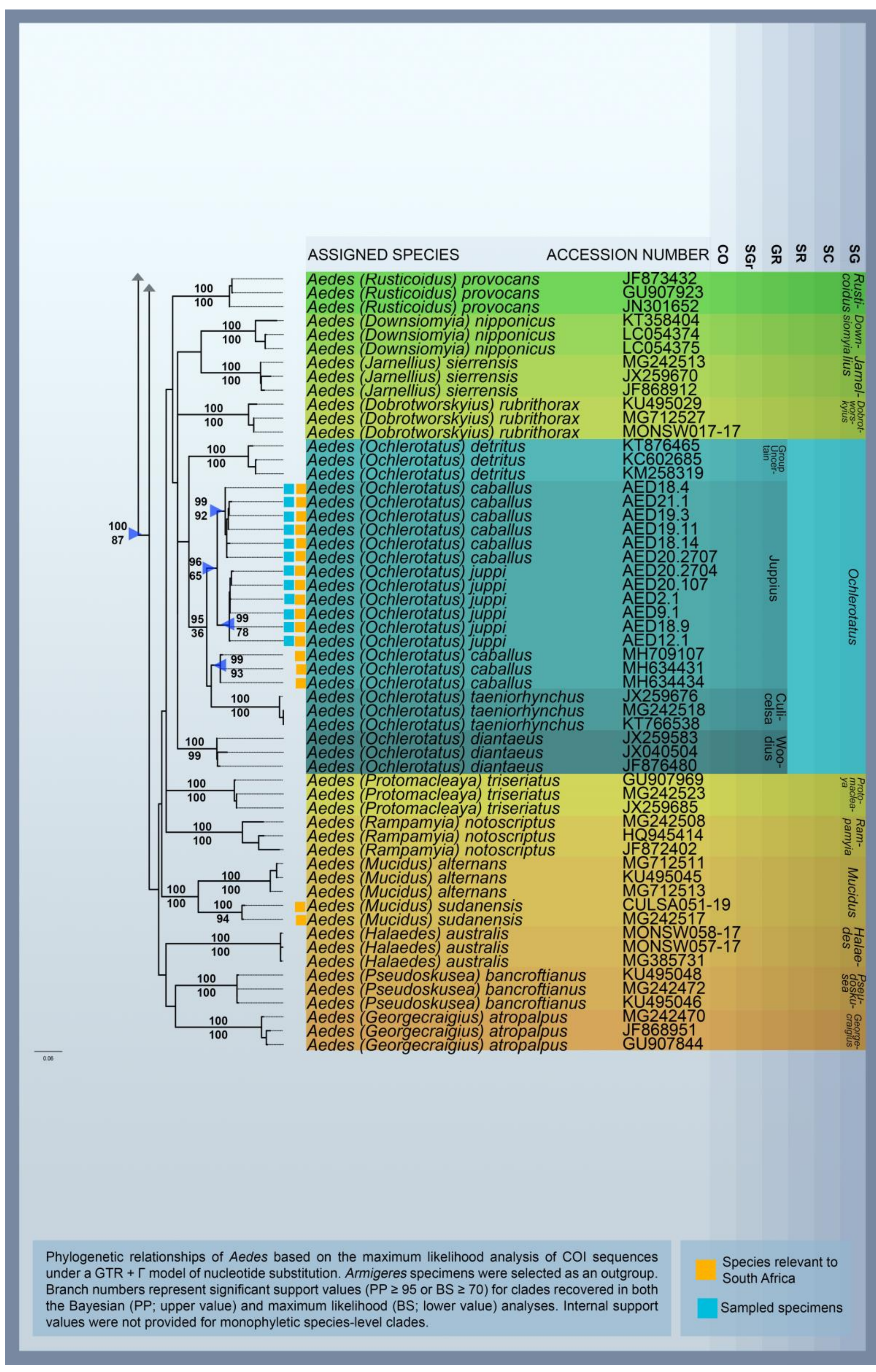


Fig. 3.7. Phylogenetic relationships of *Aedes* based on the COI nucleotide sequences.

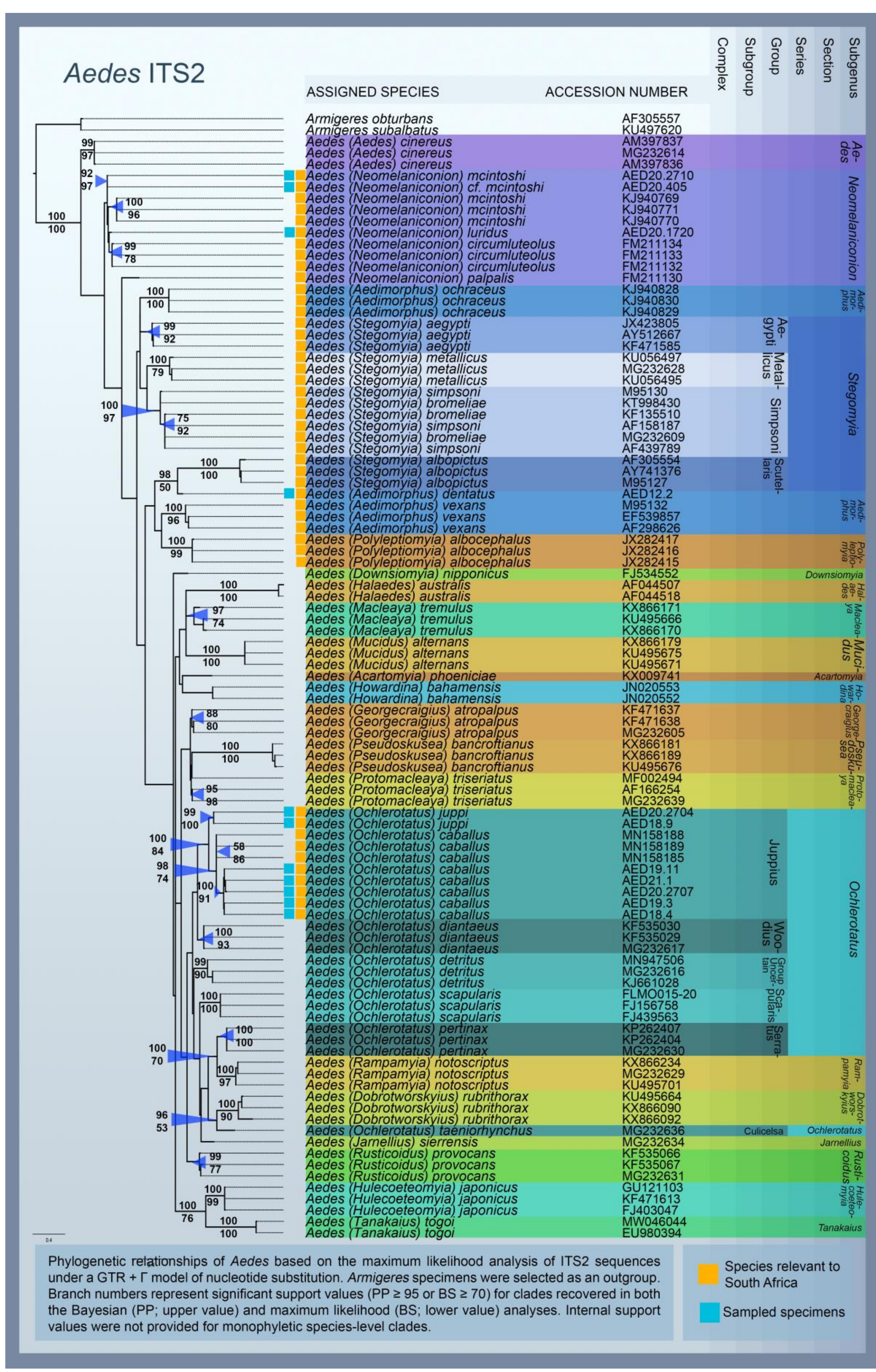


Fig. **3**.**8**. Phylogenetic relationships of *Aedes* based on the ITS2 nucleotide sequences.

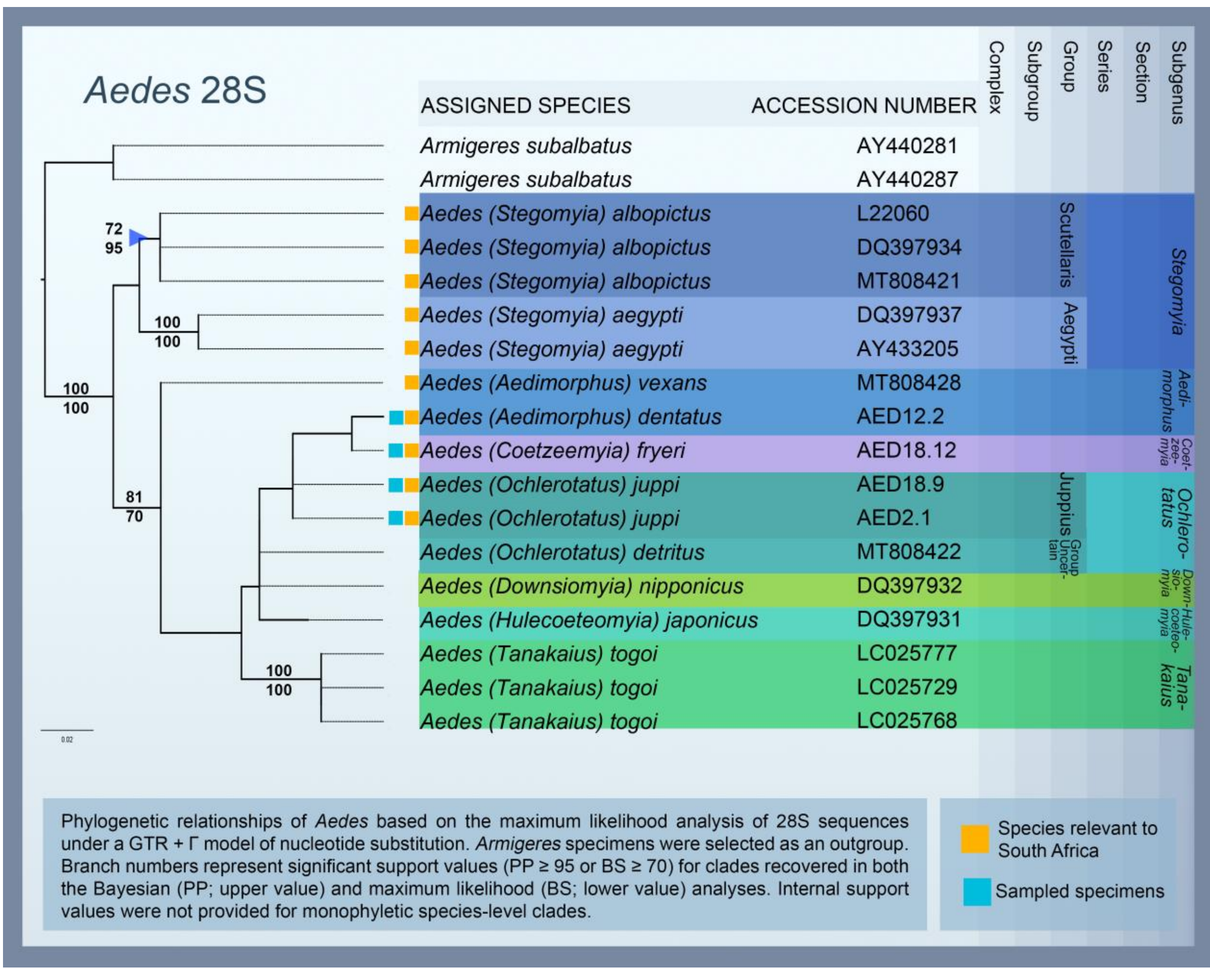


Fig. 3.9. Phylogenetic relationships of *Aedes* based on the 28S nucleotide sequences.

3.7.1.1 *AEDES* SUBGENERA WITH A COMPREHENSIVE REPRESENTATION

Four subgenera were well-represented within the public DNA databases and it was possible to include sequences of numerous species and multiple DNA regions for each taxon. This extensive taxonomic coverage provided valuable insights into the topology and monophyly of the included taxa, in addition to the stability of their affiliations across multiple DNA regions.

Subgenus *Aedimorphus*

Aedimorphus was exclusively represented by South African species, including sampled individuals of *Ae*. (*Adm*.) *eritreae* (AED22.16) in the COI dataset and *Ae*. (*Adm*.) *dentatus* (AED12.2) in the ITS2 and 28S datasets. *Aedimorphus* had a relatively low taxonomic coverage and few species remained consistent across datasets. However, the shared species were still included in inconsistent affiliations and no common clades were recovered between the DNA regions. Nonetheless, all analyses recovered the subgenus as polyphyletic, which may have been the product of the weak internal phylogenetic resolution.

Despite the lack of consistent specific associations, both the COI and ITS2 analyses produced scattered *Aedimorphus* clades that were situated within the vicinity of *Stegomyia*. The inclusion of *Catageiomyia* in the COI analyses similarly resulted in this subgenus' association with *Aedimorphus*. The sampled specimens were incorporated into clades consisting of *Ae*. (*Adm.*) *eritreae* (AED22.16) + *Ae*. (*Adm.*) *leesoni* + *Ae*. (*Cat.*) *tarsalis* (PP 93) in the COI analyses (Fig. 3.7) and *Ae*. (*Adm.*) *dentatus* (AED12.2) + *Ae*. (*Stg.*) *albopictus* + *Ae*. (*Adm.*) *vexans* + *Ae*. (*Polyleptiomyia*) *albocephalus* in the ITS2 analyses (Fig. 3.8). However, these affiliations were not corroborated by multiple DNA regions, since *Ae*. (*Adm.*) *eritreae* was only represented in the COI dataset and *Ae*. (*Adm.*) *dentatus'* relationship with *Stegomyia* was not recovered in the 28S analyses, where it was sister to *Coetzeemyia* (*Ae*. *fryeri*) (Fig. 3.9).

Subgenus *Mucidus*

Mucidus was represented by two species in the COI dataset, including *Ae*. (*Muc.*) *sudanensis* as a South African species. The COI results produced a well-supported monophyletic clade for *Mucidus* (Fig. 3.7; PP 100, BS 100), while additional relationships were poorly resolved. However, the subgenus was only represented by *Ae*. (*Muc.*) *alternans* in the ITS2 analyses (Fig. 3.8), which formed an unsupported *Mucidus* + *Halaedes* + *Macleaya* clade in the maximum likelihood (ML) analysis. Thus, no affiliations of *Mucidus* remained consistent between the DNA regions.

Subgenus *Neomelaniconion*

The subgenus *Neomelaniconion* was entirely represented by species relevant to South Africa, and was included in the COI and ITS2 datasets. *Aedes* (*Neo.*) *unidentatus* (AED22.18, AED8.30), *Ae*. (*Neo.*) *luridus* (AED20.1720) and *Ae*. (*Neo.*) *luteolateralis* (AED22.28) consisted of sampled specimens, while *Ae*. (*Neo.*) *mcintoshi* was represented by generated (AED20.2710, AED20.405, AED22.19) and publicly available sequences. The placement of clustered *Neomelaniconion* species in relation to closely allied taxa was inconsistent between analyses, likely due to the differing datasets.

The COI analyses produced a monophyletic *Neomelaniconion* clade (PP 100, BS 94), which was sister to the subgenus *Aedes* (Fig. 3.10 a). Here, both *Ae*. (*Neo.*) *mcintoshi* and *Ae*. (*Neo.*) *unidentatus* were polyphyletic. *Aedes* (*Neo.*) *mcintoshi* was associated with three different species (*Ae*. *circumluteolus*, *Ae*. *luteolateralis* and *Ae*. *unidentatus*), while *Ae*. (*Neo.*) *unidentatus* was additionally associated with *Ae*. (*Neo.*) *luridus*. Here, the *Ae*. (*Neo.*) cf. *mcintoshi* specimen with light coloured sterna (AED20.405; Fig. 3.6 c) grouped with *Ae*. (*Neo.*) *luteolateralis*. The groupings of these species were not associated with their geography or morphology, since *Ae*. (*Neo.*) *mcintoshi* was not stratified by its region of origin, while the two morphologically identical *Ae*. (*Neo.*) *unidentatus* specimens were not closely related.

Both ITS2 analyses produced a *Neomelaniconion* cluster that was situated within the vicinity of the subgenus *Aedes*, which consisted of unsupported or poorly resolved clades. Most *Neomelaniconion*

species were either included in a single ML clade (Fig. 3.10 b) or spread across several Bayesian polytomies (results not shown). Additionally, both ITS2 analyses recovered a distant and terminally placed *Ae.* (*Neo.*) *palpalis*, which was not included in the COI dataset. The ITS2 analyses produced more coherent species-level groupings, including the separation of Kenyan (PP 100, BS 96) and sampled (PP 92, BS 97; AED20.2710, AED20.405) *Ae.* (*Neo.*) *mcintoshi* lineages. Here, the atypical specimen AED20.405 grouped with conspecific sampled specimens. The two geographically distinct *Ae.* (*Neo.*) *mcintoshi* lineages once again resulted in the non-monophyly of the species, as recovered in the COI results, while no consistent interspecific relationships were recovered between the DNA regions. Since the representative species differed between the datasets, the relationships of *Ae.* (*Neo.*) *luridus*, *Ae.* (*Neo.*) *luteolateralis*, *Ae.* (*Neo.*) *unidentatus* and *Ae.* (*Neo.*) *palpalis* could not be confirmed.

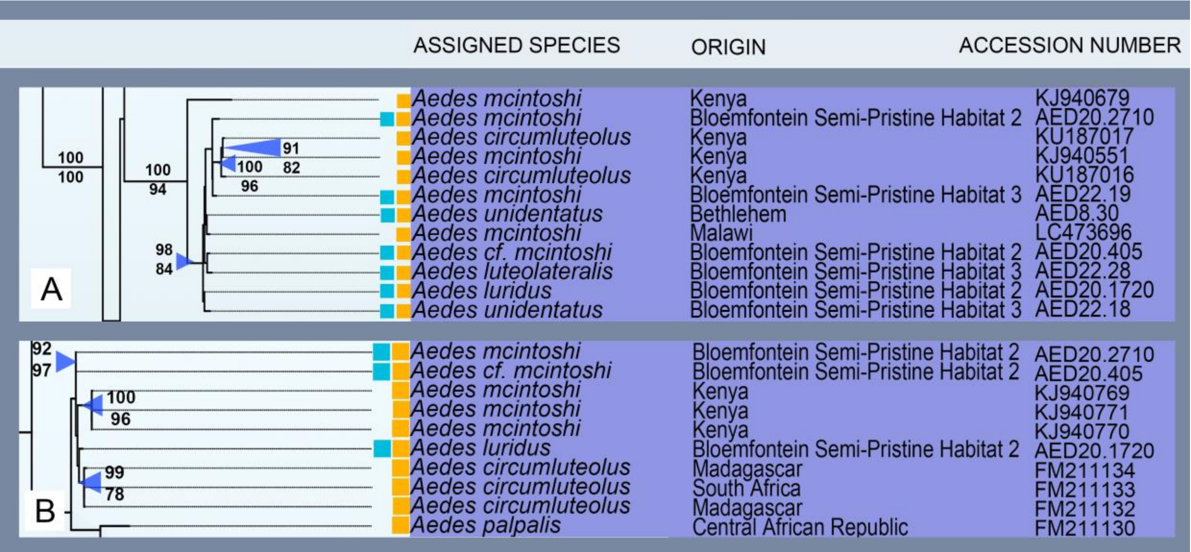


Fig. 3.10. The relationships of *Neomelaniconion* species based on the maximum likelihood analyses: a) COI; b) ITS2. Figures represent the relevant subsets of Fig. 3.7 and 3.8. Numbers represent significant support values (PP ≥ 95 or BS ≥ 70) for clades recovered in both the Bayesian (PP; top value) and maximum likelihood (BS; bottom value) analyses.

Subgenus *Ochlerotatus*

A relatively large number of *Ochlerotatus* species were included in the datasets, where two of the South African species, *Ae.* (*Och*) *juppi* and *Ae.* (*Och.*) *caballus* consisted of numerous sampled individuals. The subgenus was non-monophyletic in all analyses and many of the inter-specific affiliations were inconsistent or poorly resolved. Nonetheless, several findings were supported by multiple DNA regions. This included the proximal placement of *Ochlerotatus* to two other subgenera, *Jarnellius* (Fig. 3.7 & 3.8) and *Downsiomyia* (Fig. 3.7 & 3.9). Its association with *Dobrotworskyius* was also recovered in the COI ML and ITS2 analyses, which resulted in a sister relationship between *Ae.* (*Och.*) *taeniorhynchus* and *Dobrotworskyius* in the ITS2 results (Fig. 3.8; PP 96). However, these clades were generally unsupported and the true association between these subgenera was likely obscured by the low internal phylogenetic signal. Nonetheless, the consistent affiliation between these taxa may still point to their shared ancestry. Multiple analyses also recovered the generally well-supported clade of the Juppius

Group, where it was monophyletic in the ITS2 results (Fig. 3.8; PP 100, BS 84) and paraphyletic with the inclusion of the Culicelsa Group in the COI analyses (Fig. 3.7; PP 95, BS 36). The Woodius Group was also regularly situated within the vicinity of the Juppius Group (Fig. 3.7 & 3.8) with a weak degree of support.

The Juppius Group was represented by two South African species, namely *Ae.* (*Och.*) *caballus* and *Ae.* (*Och.*) *juppi*, which were closely associated in all relevant analyses (Fig. 3.11 a, b). *Aedes* (*Och.*) *caballus* consisted of separate lineages for South African and Iranian specimens, which resulted in its polyphyletic arrangement in the COI analyses. Here, South African *Ae.* (*Och.*) *caballus* specimens were sister to *Ae.* (*Och.*) *juppi* (PP 96, BS 65), while Iranian specimens were sister to the Culicelsa Group (Fig. 3.11 a; PP 94). Conversely, *Ae.* (*Och.*) *caballus* was recovered as a monophyletic grouping in the ITS2 results (Fig. 3.11 b; PP 98, BS 74).

The Juppius Group was also represented by numerous sampled individuals. The sampled *Ae.* (*Och.*) *caballus* (AED18.4, AED18.14, AED19.3, AED19.11, AED20.2707, AED21.1) and *Ae.* (*Och.*) *juppi* (AED2.1, AED9.1, AED12.1, AED18.9, AED20.107, AED20.2704) specimens all formed individual well-supported monophyletic groupings in the COI (Fig. 3.11 a) and ITS2 (Fig. 3.11 b) analyses. The COI dataset included six morphologically similar sampled *Ae.* (*Och.*) *juppi* individuals from Bloemfontein, Bethulie and Memel. These results yielded no consistent or well-supported intraspecific groupings or stratification of the specimens based on their geography. The COI dataset similarly included six sampled *Ae.* (*Och.*) *caballus* specimens from four different Bloemfontein sites. Most *Ae.* (*Och.*) *caballus* groupings were not consistent between the phylogenetic methods and were not associated with their sampling localities or morphology. One morphologically divergent specimen (AED18.4; Fig. 3.6 e) consisted of a relatively large male with LMS that was verified as a member of *Ae.* (*Och.*) *caballus* through DNA sequence identification. The specimen likewise grouped securely with the sampled conspecific specimens in the COI phylogenetic results.

Unlike the COI results, the internal groupings of both species were consistent in the two ITS2 analyses, where all sampled specimens were collected at Bloemfontein. The large *Ae.* (*Och.*) *caballus* male (AED18.4) once again grouped with the other sampled conspecific specimens, while all *Ae.* (*Och.*) *juppi* specimens were morphologically similar. Therefore, the topology of both species was not associated with any morphological variation. Finally, the 28S dataset included two *Ae.* (*Och.*) *juppi* specimens from Bloemfontein (AED2.1, AED18.9), which formed a monophyletic grouping in the Bayesian results (results not shown) and clustered together in the ML tree (Fig. 3. 11 c).

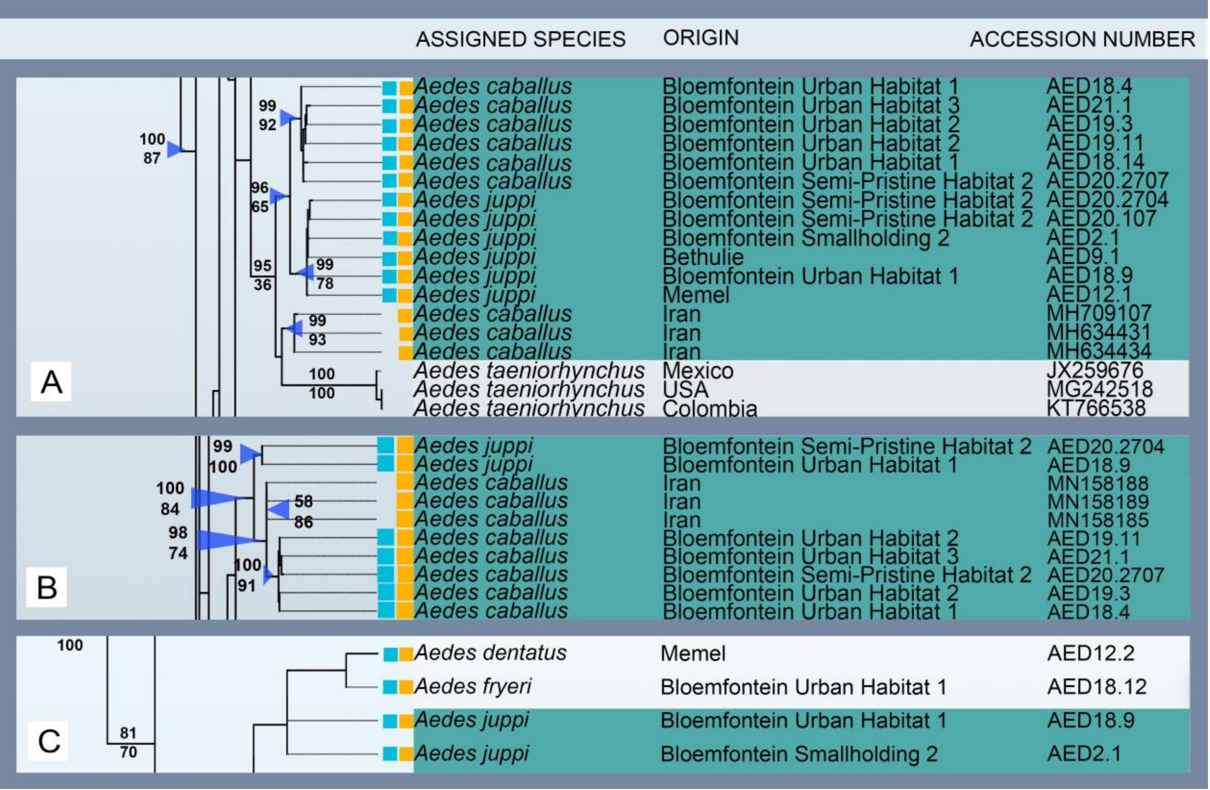


Fig. 3.11. The relationships of the Juppius Group based on the maximum likelihood analyses: a) COI; b) ITS2; c) 28S. Figures represent the relevant subsets of Fig. 3.7, 3.8 and 3.9. Numbers represent significant support values (PP ≥ 95 or BS ≥ 70) for clades recovered in both the Bayesian (PP; upper value) and maximum likelihood (BS; lower value) analyses.

Subgenus *Stegomyia*

Stegomyia was represented by seven South African species, including sampled individuals of *Ae*. (*Stg.*) *aegypti* (AED14.1.1, AED18.11) and *Ae*. (*Stg.*) *contiguus* (AED9.2). The subgenus was polyphyletic in the COI and ITS2 analyses (Fig. 3.7 & 3.8), while the two species in the 28S analyses formed a weakly supported monophyletic grouping (Fig. 3.9). The numerous polyphyletic *Stegomyia* clades were interspersed with *Aedimorphus* clades in both the COI and ITS2 results, with the addition of scattered *Catageiomyia* clades in the COI analyses. The clades of these three polyphyletic subgenera were situated in close proximity to each other, although groupings were generally not well-supported. These affiliations may have reflected a distant shared history that has been obscured by substitutional saturation.

Due to the weak internal resolution, *Stegomyia*'s topology was generally unsupported and unresolved in the COI and ITS2 analyses. However, several relationships were still recovered by multiple gene regions. Here, both the COI and 28S analyses recovered a clade consisting of the Aegypti + Scutellaris Groups, which was only well supported in the COI Bayesian results (PP 95). However, this grouping was not recovered in the ITS2 results. Additionally, the Simpsoni Group was represented in the COI and ITS2 datasets, where it formed a well-supported monophyletic grouping (COI: PP100, BS 100; ITS2: PP 100, BS 97), yet its representatives (*Ae. simpsoni* & *Ae. bromeliae*) were individually

recovered as non-monophyletic in multiple analyses (Fig. 3.12 a, b). In the COI ML analysis, *Ae.* (*Stg.*) *simpsoni* was non-monophyletic, while both species were polyphyletic in the ITS2 results. The specimens were partly stratified by their region of origin, since the two Ugandan *Ae.* (*Stg.*) *bromeliae* specimens formed a single clade in the COI analyses, while neither of the two Ugandan *Ae.* (*Stg.*) *simpsoni* or two East African (Kenya & Uganda) *Ae.* (*Stg.*) *bromeliae* specimens were grouped together in the ITS2 results.

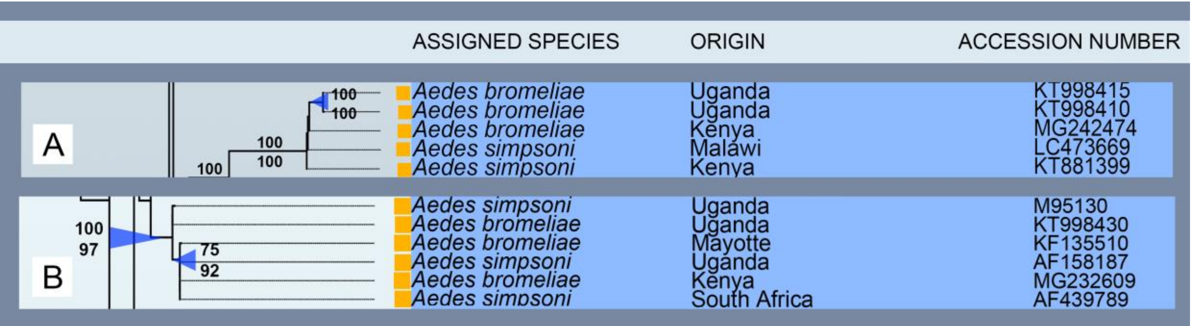


Fig. **3.12**. The relationships of the Simpsoni Group based on the maximum likelihood analyses: a) COI; b) ITS2. Figures represent the relevant subsets of Fig. 3.7 and 3.8. Numbers represent significant support values (PP ≥ 95 or BS ≥ 70) for clades recovered in both the Bayesian (PP; upper value) and maximum likelihood (BS; lower value) analyses.

The Simpsoni Group was also frequently affiliated with the Metallicus Group, where the taxa shared a clade in the ITS2 ML (Fig. 3.8) and COI (Fig. 3.7) analyses, with the addition of the Poweri Group in the COI results. Here, both the Simpsoni + Metallicus + Poweri Group clade (PP 100, BS 81) and the Metallicus + Poweri Group clade (PP 100, BS 88) were well-supported.

In the COI dataset, *Ae.* (*Stg.*) *aegypti* was also represented by two morphologically similar sampled individuals (AED14.1.1, AED18.11). Here, the internal topology was conserved amongst the two phylogenetic methods. The grouping between the Bothaville and Chilean specimens was well-supported (PP 94, BS 90), while the second unsupported grouping consisted of the Bloemfontein and laboratory colony (Liverpool strain) specimens (Fig. 3.13). Therefore, these groupings were not associated with their geography or morphology.

Fig. **3.13**. The relationships of *Ae.* (*Stg.*) *aegypti* based on the COI maximum likelihood analyses. The figure represents the relevant subset of Fig. 3.7. Numbers represent significant support values (PP ≥ 95 or BS ≥ 70) for clades recovered in both the Bayesian (PP; upper value) and maximum likelihood (BS; lower value) analyses.

3.7.1.2 *AEDES* SUBGENERA WITH A LIMITED REPRESENTATION

Numerous subgenera were poorly represented within the public DNA databases, and fewer sequences were available for the ITS2 and 28S DNA regions. Therefore, these subgenera were often represented by a single species or DNA region within the current datasets, thus limiting the utility of the generated results. However, these taxa were frequently underrepresented within the literature and the current phylogenetic examinations still provided the opportunity to gain novel insights into their evolutionary history and phylogenetic relationships.

Numerous subgenera were represented by a single species within a single dataset, and were often affiliated with the larger subgenera within the analyses. These subgenera included *Acartomyia*, *Coetzeemyia*, *Diceromyia*, *Fredwardsius* and *Polyleptiomyia*. The COI datasets included *Ae.* (*Dic.*) *furcifer* and *Ae.* (*Fre.*) *vittatus* as South African representatives of *Diceromyia* and *Fredwardsius*. The affiliations of both subgenera were not consistent or well-supported, except for *Diceromyia*'s constant placement as the most basal clade within the genus (Fig. 3.7). Within the ITS2 dataset, *Polyleptiomyia* was represented by a single South African species, *Ae.* (*Pmt.*) *albocephalus*. Both ITS2 analyses recovered a clade consisting of *Polyleptiomyia* + *Ae.* (*Stg.*) *albopictus* + *Ae.* (*Adm.*) *dentatus* + *Ae.* (*Adm.*) *vexans* (Fig. 3.8), although these relationships were unsupported. No public data was available for *Coetzeemyia* and the subgenus was only represented by a single sampled individual (*Ae. fryeri*; AED18.12). The specimen was successfully sequenced for the 28S region, where the analyses recovered an unsupported clade consisting of *Ae.* (*Coe.*) *fryeri* + *Ae.* (*Adm.*) *dentatus*, while additional affiliations were inconsistent between the analyses (Fig. 3.9).

Another set of subgenera were represented by a single species, yet were included within multiple datasets, thus providing some insight into the stability of the taxon's placement. Many of these subgenera were associated with one another across multiple analyses, while *Halaedes* and *Protomacleaya* did not produce any consistent or well-supported relationships in the COI and ITS2 analyses (Fig. 3.7 & 3.8).

One cluster of weakly associated subgenera included *Dobrotworskyius*, *Jarnellius*, *Rampamyia* and *Rusticoidus*, each represented by a single species and affiliated with *Ochlerotatus* in the COI and ITS2 analyses (Fig. 3.7 & 3.8). The relationships of these subgenera were often unresolved in the Bayesian results, except for *Jarnellius*, which consistently shared a small clade with several *Ochlerotatus* species. In both ML analyses, *Ae.* (*Dob.*) *rubrithorax*, *Ae.* (*Jar.*) *sierrensis*, *Ae.* (*Ram.*) *notoscriptus and Ae.* (*Rus.*) *provocans* shared an unsupported clade with *Ochlerotatus* (Fig. 3.7 & 3.8), while the only well-supported relationships consisted of *Rampamyia* + *Ae.* (*Och.*) *pertinax* (PP 100, BS 70) and *Dobrotworskyius* + *Ae.* (*Och.*) *taeniorhynchus* (PP 96) in the ITS2 results. However, no specific interactions remained constant between the DNA regions.

A few other subgenera were also associated with *Ochlerotatus* across multiple analyses. This included *Downsiomyia*, which was represented by *Ae*. (*Dow.*) *nipponicus* in all datasets. Both COI analyses recovered an unsupported clade consisting of *Downsiomyia* + *Jarnellius*, which was in turn associated with a larger *Ochlerotatus* (in part) grouping (Fig. 3.7). The affiliations of *Downsiomyia* were also unresolved and unsupported in the ITS2 analyses (Fig. 3.8), while the subgenus shared a clade with *Hulecoeteomyia* and *Ochlerotatus* in the 28S analyses (Fig. 3.9).

The association between *Hulecoeteomyia*, *Tanakaius* and *Ochlerotatus* was also recovered by multiple DNA regions. *Hulecoeteomyia* and *Tanakaius* were represented by *Ae*. (*Hul.*) *japonicus* and *Ae*. (*Tan.*) *togoi* in all three datasets. Their relationships were not well resolved in the COI analyses and both taxa shared a mutual clade with several other subgenera in the ML analysis, including *Ochlerotatus* (Fig. 3.7). On the other hand, the ITS2 analyses recovered a clade consisting solely of *Hulecoeteomyia* + *Tanakaius*, with a significant degree of support (Fig. 3.8; PP 100, BS 76). Lastly, the 28S analyses placed *Tanakaius* as a sister group to an unsupported clade consisting of several subgenera, including *Ochlerotatus* and *Hulecoeteomyia* (Fig. 3.9). Although these 28S relationships were not well-supported, this affiliation between *Tanakaius*, *Hulecoeteomyia* and *Ochlerotatus* was recovered by multiple DNA regions.

Another consistent affiliation included the association between *Georgecraigius* and *Pseudoskusea*, where the subgenera were represented by *Ae*. (*Grg.*) *atropalpus* and *Ae*. (*Psk.*) *bancroftianus* in the COI and ITS2 datasets. These relationships were mainly unresolved in the Bayesian results, while the COI ML results recovered a clade consisting of *Georgecraigius* + *Pseudoskusea* (Fig. 3.7). Both ITS2 analyses also recovered a clade involving *Georgecraigius* and *Protomacleaya*, with the addition of *Pseudoskusea* in the ML analysis (Fig. 3.8). Therefore, this affiliation between *Georgecraigius* and *Pseudoskusea* was recovered by multiple analyses.

Furthermore, the subgenus *Aedes* was represented by *Ae*. (*Aed.*) *cinereus* in the COI and ITS2 datasets, where it was consistently affiliated with *Neomelaniconion*. Both COI analyses produced a clade consisting of *Aedes* + *Neomelaniconion* (Fig. 3.7), which had a moderate degree of support in the Bayesian analysis (PP 94). Both *Aedes* and *Neomelaniconion* were also situated proximally to one another in the ITS2 analyses, where *Aedes* was placed basally to *Neomelaniconion* (Fig. 3.8).

The final cluster of subgenera consisted of *Acartomyia*, *Howardina* and *Macleaya*. *Howardina* and *Macleaya* were represented by *Ae*. (*How.*) *bahamensis* and *Ae.* (*Mac.*) *tremulus* in the COI and ITS2 datasets, while *Acartomyia* was only represented by *Ae*. (*Acy.*) *phoeniciae* in the ITS2 dataset. Both COI analyses recovered a clade including *Howardina*, *Macleaya* and *Ae*. (*Och.*) *scapularis* (PP 93), with the addition of two additional subgenera in the ML results (Fig. 3.7). However, the ITS2 results produced an unsupported clade containing *Acartomyia* + *Howardina* (Fig. 3.8), which was in turn affiliated with a small ML clade containing *Macleaya*. Therefore, both DNA regions yielded a weak association between *Howardina* and *Macleaya*.

In contrast to the previously mentioned subgenera, *Catageiomyia* was represented by multiple species, yet was only included within a single dataset. The subgenus was exclusively represented by South African species in the COI analyses, including a single sampled specimen of *Ae*. (*Cat*.) *mixtus* (AED4.2.2). *Catageiomyia* was polyphyletic and affiliated with the subgenus *Aedimorphus* in both COI analyses (Fig. 3.7), resulting in two well-supported clades of *Ae*. (*Cat*.) *tarsalis* + *Ae*. (*Adm*.) *leesoni* (PP 100, BS 90) and *Ae*. (*Cat*.) *mixtus* + *Ae*. (*Cat*.) *argenteopunctatus* (PP 100, BS 97).

3.7.2 *CULEX*

Culex was represented by 11 subgenera, 51 species and 254 sequences across the three datasets, and consisted of two sections, one series and 23 groups. The dataset contained 21 South African species, including 45 generated sequences originating from five species. The identity of most sampled *Culex* specimens were supported by a consensus of conspecific matches during DNA sequence identification. However, the identity of two sampled specimens could not be verified with a BLAST query. One sampled *Cx*. (*Cux*.) *univittatus* (CLX2.243) specimen was only represented by an ITS2 sequence, a region not included in the GenBank database for this species. Nonetheless, the BLAST results of the ITS2 region were homologous to the ITS2 matches of other conspecific specimens. Furthermore, *Cx*. (*Mai*.) *salisburiensis* (CLX9.2) was not represented in the GenBank database, yet the query produced a relatively close match (91.11%) to a *Cx*. (*Mai*.) *hortensis* specimen from Algeria, a species within the same subgenus.

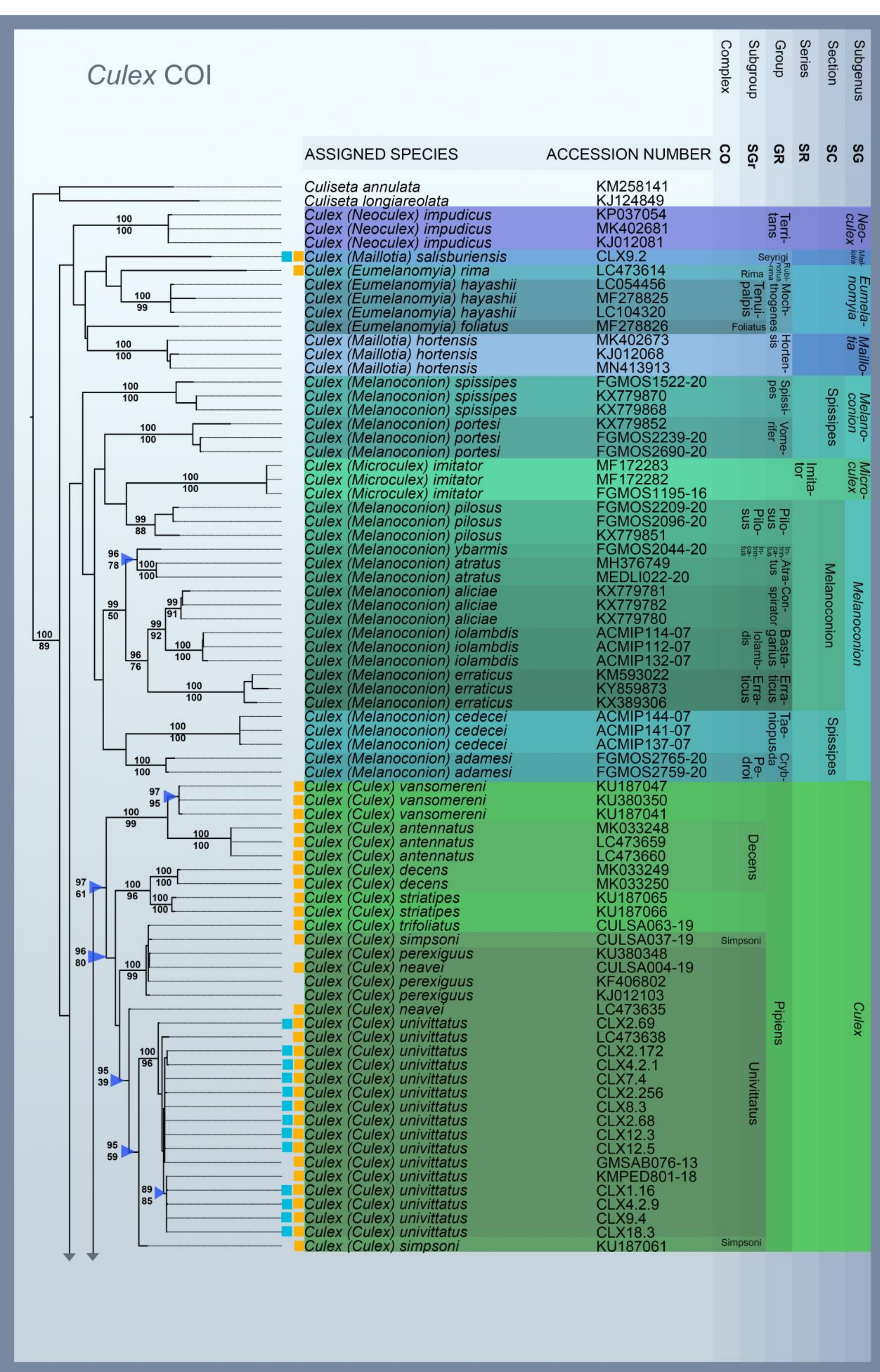
The COI dataset represented the greatest taxonomic coverage and consisted of 11 subgenera and 50 species. The alignment consisted of 658 sites, including 408 conserved, 250 variable and 230 parsimony informative positions. These analyses produced relatively well-resolved internal relationships, with few multifurcations in the Bayesian analysis (results not shown) and many well-supported branches in the ML analysis (Fig. 3.14). Nonetheless, support was generally greater for terminal relationships, as compared to internal groupings, while numerous clades remained consistent between the phylogenetic methods. Here, the larger subgenera, such as *Culex*, *Melanoconion* and *Oculeomyia*, were rendered polyphyletic or paraphyletic by smaller subgenera.

Fewer sequences were available for the ITS2 region, where the dataset represented five subgenera and 26 species. The alignment consisted of 458 sites, including 101 conserved, 333 variable and 310 parsimony informative positions. Clade support was inconsistent between analyses, yet aspects of the topology remained relatively consistent between the phylogenetic methods (Fig. 3.15). This included the structure of the subgenus *Culex*, which was consistently non-monophyletic and associated with numerous smaller subgenera.

The 28S dataset was relatively small and represented three subgenera and seven species. The alignment consisted of 416 sites, including 372 conserved, 40 variable and 28 parsimony informative

positions. In the 28S analyses, internal relationships were generally well-resolved, while the results recovered minimal phylogenetic distinction between closely related species (Fig. 3.16). Here, both phylogenetic methods yielded similar topologies. Despite the limited taxonomic coverage, the largest subgenus was once again recovered as non-monophyletic, while a single sequence represented each of the remaining two subgenera.

Multiple findings were shared between the DNA regions, while other interactions were only represented by a single dataset. The corroborated results included the non-monophyly of the subgenus *Culex* and the inclusion of *Barraudius* and *Oculeomyia* within its overarching clade. The scattered groupings of *Culex* also resulted in the polyphyly of its Pipiens Group, while two of its species, *Cx*. (*Cux*.) *pipiens* and *Cx*. (*Cux*.) *quinquefasciatus* were frequently recovered as polyphyletic. The analyses also recovered clades consisting of the Vishnui + Sitiens Subgroups and the Theileri + Univittatus Subgroups.



Culex COI
ASSIGNED SPECIES
ACCESSION NUMBER
CO
SGr
GR
SR
SC
SG
Complex
Subgroup
Group
Series
Section
Subgenus
Culiseta annulata KM258141
Culiseta longiareolata KJ124849
Culex (Neoculex) impudicus KP037054
Culex (Neoculex) impudicus MK402681
Culex (Neoculex) impudicus KJ012081
Culex (Maillotia) salisburiensis CLX9.2
Culex (Eumelanomyia) rima LC473614
Culex (Eumelanomyia) hayashii LC054456
Culex (Eumelanomyia) hayashii MF278825
Culex (Eumelanomyia) hayashii LC104320
Culex (Eumelanomyia) foliatus MF278826
Culex (Maillotia) hortensis MK402673
Culex (Maillotia) hortensis KJ012068
Culex (Maillotia) hortensis MN413913
Culex (Melanoconion) spissipes FGMOS1522-20
Culex (Melanoconion) spissipes KX779870
Culex (Melanoconion) spissipes KX779868
Culex (Melanoconion) portesi KX779852
Culex (Melanoconion) portesi FGMOS2239-20
Culex (Melanoconion) portesi FGMOS2690-20
Culex (Microculex) imitator MF172283
Culex (Microculex) imitator MF172282
Culex (Microculex) imitator FGMOS1195-16
Culex (Melanoconion) pilosus FGMOS2209-20
Culex (Melanoconion) pilosus FGMOS2096-20
Culex (Melanoconion) pilosus KX779851
Culex (Melanoconion) ybarmis FGMOS2044-20
Culex (Melanoconion) atratus MH376749
Culex (Melanoconion) atratus MEDLI022-20
Culex (Melanoconion) aliciae KX779781
Culex (Melanoconion) aliciae KX779782
Culex (Melanoconion) aliciae KX779780
Culex (Melanoconion) iolambdis ACMIP114-07
Culex (Melanoconion) iolambdis ACMIP112-07
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Culex (Melanoconion) erraticus KM593022
Culex (Melanoconion) erraticus KY859873
Culex (Melanoconion) erraticus KX389306
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Culex (Melanoconion) adamesi FGMOS2765-20
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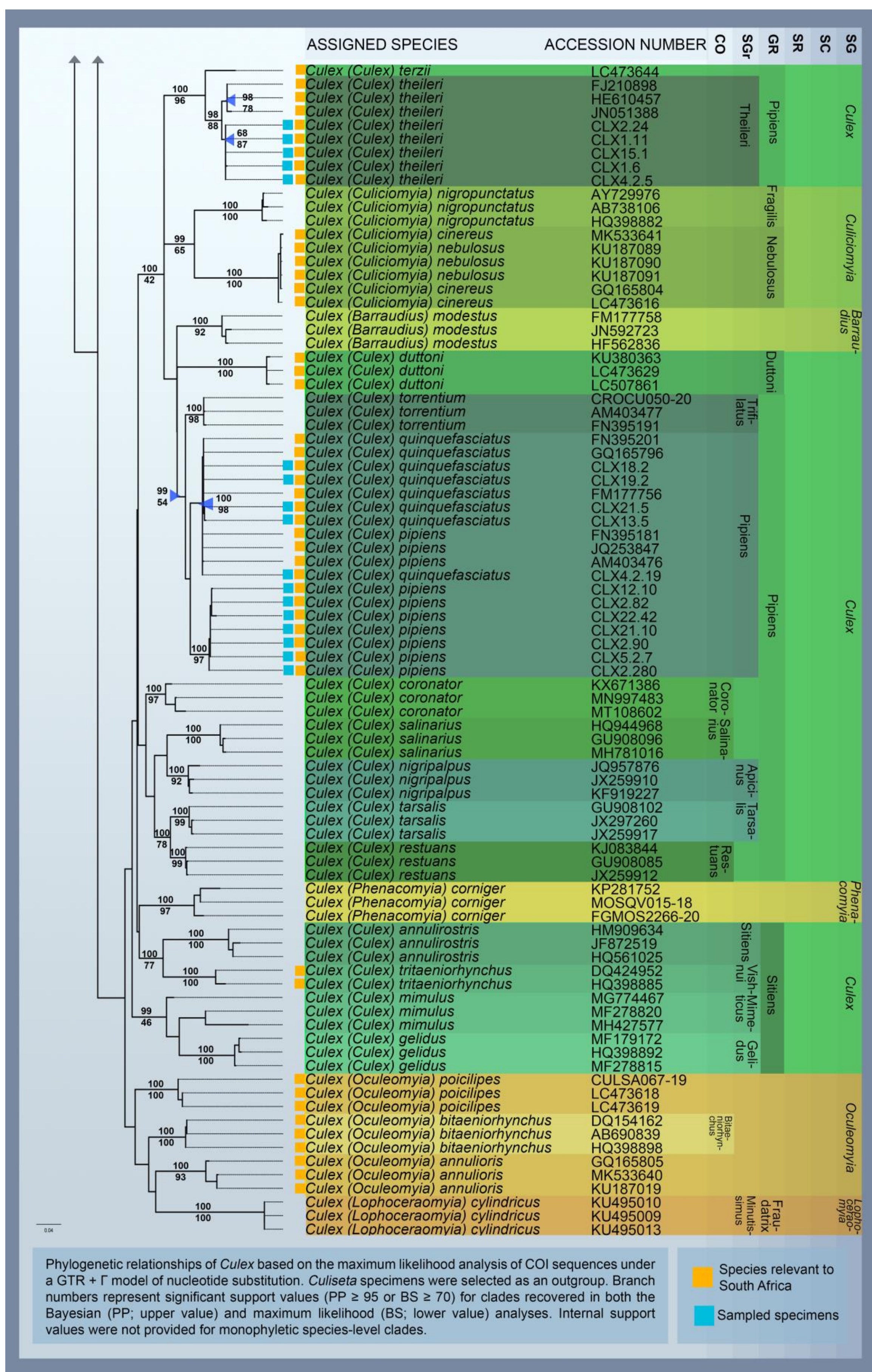
Culex (Culex) vansomereni KU187047
Culex (Culex) vansomereni KU380350
Culex (Culex) vansomereni KU187041
Culex (Culex) antennatus MK033248
Culex (Culex) antennatus LC473659
Culex (Culex) antennatus LC473660
Culex (Culex) decens MK033249
Culex (Culex) decens MK033250
Culex (Culex) striatipes KU187065
Culex (Culex) striatipes KU187066
Culex (Culex) trifoliatus CULSA063-19
Culex (Culex) simpsoni CULSA037-19
Culex (Culex) perexiguus KU380348
Culex (Culex) neavei CULSA004-19
Culex (Culex) perexiguus KF406802
Culex (Culex) perexiguus KJ012103
Culex (Culex) neavei LC473635
Culex (Culex) univittatus CLX2.69
Culex (Culex) univittatus LC473638
Culex (Culex) univittatus CLX2.172
Culex (Culex) univittatus CLX4.2.1
Culex (Culex) univittatus CLX7.4
Culex (Culex) univittatus CLX2.256
Culex (Culex) univittatus CLX8.3
Culex (Culex) univittatus CLX2.68
Culex (Culex) univittatus CLX12.3
Culex (Culex) univittatus CLX12.5
Culex (Culex) univittatus GMSAB076-13
Culex (Culex) univittatus KMPED801-18
Culex (Culex) univittatus CLX1.16
Culex (Culex) univittatus CLX4.2.9
Culex (Culex) univittatus CLX9.4
Culex (Culex) univittatus CLX18.3
Culex (Culex) simpsoni KU187061
Neoculex
Territans
Maillotia
Seyrigi
Eumelanomyia
Rima
Rubinotus
Tenuipalpis
Mochthogenes
Foliatus
Maillotia
Hortensis
Melanoconion
Spissipes
Spissipes
Vomerifer
Microculex
Imitator
Pilosus
Pilosus
Intrincatus
Atratus
Conspirator
Bastagarius
Iolambdis
Erraticus
Erraticus
Melanoconion
Taeniopus
Crybda
Pedroi
Spissipes
Decens
Simpsoni
Pipiens
Univittatus
Simpsoni
Culex



Phylogenetic relationships of *Culex* based on the maximum likelihood analysis of COI sequences under a GTR + Γ model of nucleotide substitution. *Culiseta* specimens were selected as an outgroup. Branch numbers represent significant support values (PP ≥ 95 or BS ≥ 70) for clades recovered in both the Bayesian (PP; upper value) and maximum likelihood (BS; lower value) analyses. Internal support values were not provided for monophyletic species-level clades.

Fig. 3.14. Phylogenetic relationships of *Culex* based on the COI nucleotide sequences.

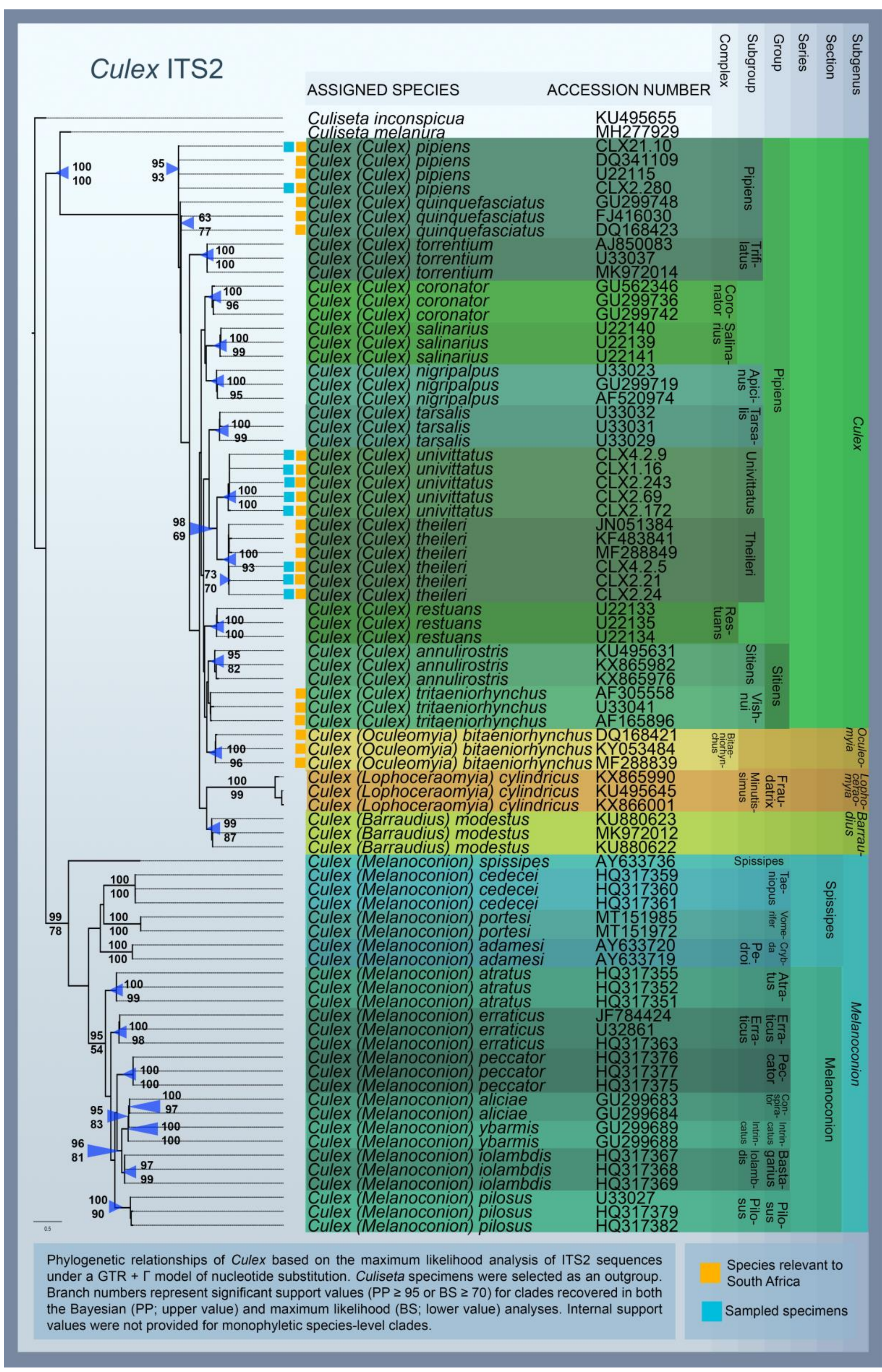


Fig. 3.15. Phylogenetic relationships of *Culex* based on the ITS2 nucleotide sequences.

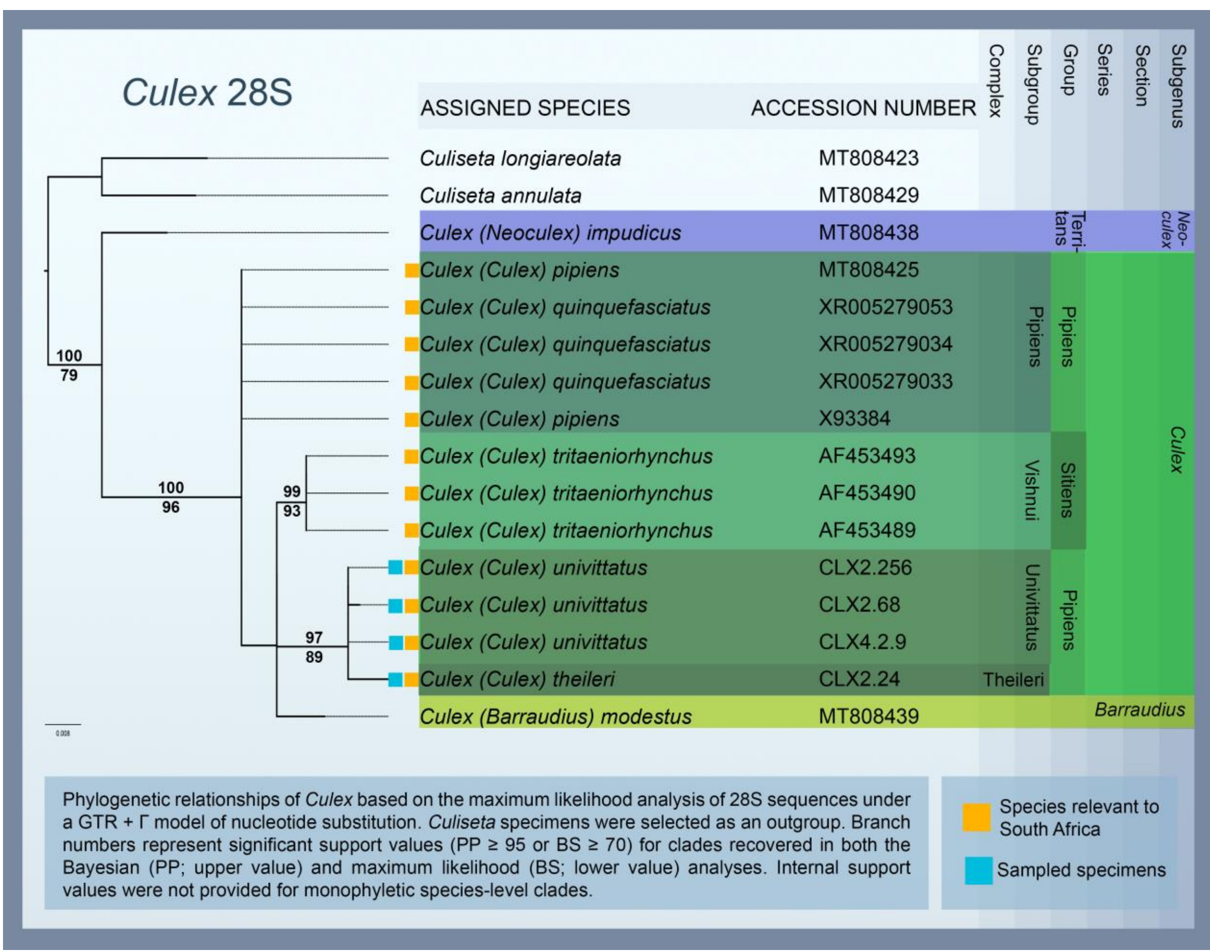


Fig. 3.16. Phylogenetic relationships of *Culex* based on the 28S nucleotide sequences.

3.7.2.1 *CULEX* SUBGENERA WITH A COMPREHENSIVE REPRESENTATION

Three of the genus' subgenera, *Culex*, *Melanoconion* and *Oculeomyia*, were all included within multiple datasets and were represented by several species. This degree of taxonomic and genetic coverage greatly improved the utility of the generated results, since it provided insight into the internal topology and stability of the taxonomic affiliations.

Subgenus *Culex*

The subgenus *Culex* was represented by many taxa, including 14 South African species and numerous sampled individuals belonging to *Cx*. (*Cux*.) *univittatus*, *Cx*. (*Cux*.) *theileri*, *Cx*. (*Cux*.) *quinquefasciatus* and *Cx*. (*Cux*.) *pipiens*. The subgenus was represented in all three datasets, where numerous affiliations were inconsistent between the DNA regions. This was partly due to the omission of numerous taxa in the ITS2 and 28S datasets, due to the lack of available sequences. The subgenus was nonetheless non-monophyletic in all analyses, with the frequent inclusion of *Barraudius* and *Oculeomyia* within the overarching clade. The subgenus *Phenacomyia* was only represented in the COI dataset, where it was similarly included within the overarching *Culex* clade (Fig. 3.14), while

Lophoceraomyia was either associated with, or incorporated within *Culex* in the COI and ITS2 results (Fig. 3.14 & 3.15). Since the subgenus was primarily represented by the Pipiens Group, this group was also polyphyletic in all analyses.

Many species were exclusively represented by the COI dataset, where these analyses produced numerous unique affiliations that could not be verified by other gene regions (Fig. 3.14). Here, the polyphyletic Univittatus Subgroup shared a clade with the Simpsoni and Decens Subgroups. Several species were also polyphyletic in the COI results, including *Cx*. (*Cux*.) *simpsoni*, *Cx*. (*Cux*.) *neavei* and *Cx*. (*Cux*.) *perexiguus*. The scattered specimens of these species were included in clades consisting of *Cx*. (*Cux*.) *simpsoni* (South Africa) + *Cx*. (*Cux*.) *neavei* (South Africa) + *Cx*. (*Cux*.) *trifoliatus* + *Cx*. (*Cux*.) *perexiguus* (PP 100, BS 99) and *Cx*. (*Cux*.) *univittatus* + *Cx*. (*Cux*.) *neavei* (Malawi) + *Cx*. (*Cux*.) *simpsoni* (Kenya) (PP 95).

The COI results also recovered the Decens Subgroup as polyphyletic, where the two species formed well-supported groupings with other members from the Pipiens Group. These clades consisted of *Cx*. (*Cux*.) *decens* (Decens Subgroup) + *Cx*. (*Cux*.) *striatipes* (PP 100, BS 96) and *Cx*. (*Cux*.) *antennatus* (Decens Subgroup) + *Cx*. (*Cux*.) *vansomereni* (PP 100, BS 99). Finally, *Cx*. (*Cux*.) *theileri* (Theileri Subgroup) formed a well-supported clade with *Cx*. (*Cux*.) *terzii* (PP 100, BS 96) and was loosely affiliated with the Pipiens and Trifilatus Subgroups in the COI analyses. However, many of these species were only represented in the COI dataset and their affiliations could not be substantiated by multiple DNA regions. The results nonetheless provided insight into the affinities of numerous South African species, including *Cx*. (*Cux*.) *antennatus*, *Cx*. (*Cux*.) *decens*, *Cx*. (*Cux*.) *neavei*, *Cx*. (*Cux*.) *simpsoni*, *Cx*. (*Cux*.) *striatipes*, *Cx*. (*Cux*.) *theileri*, *Cx*. (*Cux*.) *terzii*, *Cx*. (*Cux*.) *trifoliatus*, *Cx*. (*Cux*.) *univittatus* and *Cx*. (*Cux*.) *vansomereni*.

A few other affiliations were supported by multiple analyses, including clades consisting of the Trifilatus + Pipiens Subgroups (COI and Bayesian ITS2 results), Apicinus Subgroup + Salinarius Complex (COI and ITS2 results) and the Univittatus + Theileri Subgroups (ITS2 and 28S results). The Pipiens Subgroup represented species relevant to South Africa (*Cx. pipiens* & *Cx. quinquefasciatus*) and the subgroup was infrequently recovered as non-monophyletic. These species were mutually aggregated in all analyses, while the affiliated Trifilatus Subgroup formed a clade with the Pipiens Subgroup in the COI (Fig. 3.14; PP 99, BS 54) and Bayesian ITS2 (PP 94, results not shown) results.

There was no apparent phylogenetic distinction between the two Pipiens Subgroup species, which resulted in their polyphyly in the COI and 28S ML analyses. However, their arrangement in the 28S results was likely caused by the weak phylogenetic signal of this relatively conserved region. Their polyphyly was nonetheless pronounced in the COI results, where *Cx*. (*Cux*.) *quinquefasciatus* was intermixed with Eurasian *Cx*. (*Cux*.) *pipiens* specimens, while South African *Cx*. (*Cux*.) *pipiens* specimens were positioned separately (Fig. 3.14). In the ITS2 analyses, *Cx*. (*Cux*.) *quinquefasciatus* was also non-monophyletic, while all *Cx*. (*Cux*.) *pipiens* specimens formed a single well-supported clade

(Fig. 3.15; PP 95, BS 93). Finally, *Cx.* (*Cux.*) *pipiens* and *Cx.* (*Cux.*) *quinquefasciatus* were either polyphyletic or included in a polytomy in the 28S results (Fig. 3.16).

The groupings of the Apicinus Subgroup + Salinarius Complex and the Univittatus + Theileri Subgroups were each recovered by two separate DNA regions. This relationship between the Apicinus Subgroup and Salinarius Complex was unsupported in all relevant analyses, while the sister relationship between the Univittatus and Theileri Subgroups was inconsistent. The extensive taxonomic coverage in the COI dataset resulted in independent affiliations for each of the Univittatus and Theileri Subgroups, while the exclusion of many associated taxa resulted in these subgroups' well-supported mutual relationship in the ITS2 (Fig. 3.15; PP 98, BS 69) and 28S (Fig. 3.16; PP 97, BS 89) analyses. Here, these subgroups were each represented by a single South African species, namely *Cx.* (*Cux.*) *theileri* and *Cx.* (*Cux.*) *univittatus*.

In addition to the Pipiens Group, the subgenus was also represented by the Duttoni and Sitiens Groups. The Duttoni Group was represented by a single South African species, *Cx.* (*Cux.*) *duttoni*, in the COI dataset, where it was included in a Duttoni Group + Pipiens Subgroup + Trifilatus Subgroup + *Barraudius* clade. The Sitiens Group was represented by four taxa, including *Cx.* (*Cux.*) *tritaeniorhynchus*, which occurs in South Africa. The Sitiens Group was polyphyletic in the COI results and consisted of two separate clades: the Mimeticus + Gelidus Subgroup (PP 99) and the Vishnui + Sitiens Subgroup (PP 100, BS 77). With the smaller ITS2 dataset, only the Vishnui + Sitiens Subgroup clade was recovered in the analyses, while the Sitiens Group was represented by a single species in the 28S dataset.

Four species of *Culex* were represented by sampled individuals, including *Cx.* (*Cux.*) *univittatus*, *Cx.* (*Cux.*) *theileri*, *Cx.* (*Cux.*) *quinquefasciatus* and *Cx.* (*Cux.*) *pipiens*. One of the most well-represented species was *Cx.* (*Cux.*) *univittatus* (CLX1.16, CLX2.172, CLX2.243, CLX2.256, CLX2.68, CLX2.69, CLX4.2.1, CLX4.2.9, CLX7.4, CLX8.3, CLX9.4, CLX12.3, CLX12.5, CLX18.3), which consisted of 13 generated and three publicly available sequences in the COI dataset, five sampled individuals in the ITS2 dataset and three sampled specimens in the 28S dataset. The *Cx.* (*Cux.*) *univittatus* clade was monophyletic and well-supported in the COI (PP 100, BS 96) and ITS2 (PP 100, BS 100) analyses, while the three sampled individuals formed a well-supported clade with *Cx.* (*Cux.*) *theileri* in the 28S results (PP 97, BS 89).

The COI and ITS2 analyses (Fig. 3.17 a, b & c) produced several internal *Cx.* (*Cux.*) *univittatus* clades consisting of geographically diverse specimens. Therefore, the clades were not stratified by their region of origin, except for the relatively basal placement of the single Malawian specimen. Furthermore, a large degree of morphological variation was observed within *Cx.* (*Cux.*) *univittatus* specimens (Fig. 3.6 m-r), where the identity of all individuals was verified with BLAST queries. Most specimens displayed a unique set of characters, which mostly did not coincide with their phylogenetic groupings. The only exception was specimen CLX2.69, a relatively dark specimen with a shorter stripe on the hind femur

(Fig. 3.6 q). This individual was the most basal *Cx*. (*Cux*.) *univittatus* specimen in the COI results, and was placed separately from other South African specimens. However, this specimen grouped securely within the South African *Cx*. (*Cux*.) *univittatus* clade in the ITS2 results.

The associated species, *Cx*. (*Cux*.) *theileri*, was represented by morphologically diverse sampled individuals (Fig. 3.6 k, l; CLX1.11, CLX1.6, CLX2.21, CLX2.24, CLX4.2.5, CLX15.1), where the identity of all specimens was supported by DNA sequence identifications. All analyses produced separate lineages for the South African and Eurasian specimens, where the species-clade was well-supported (COI: PP 98, BS 88; ITS2: PP 100, BS 93). The separate South African (BS 87) and Eurasian lineages (PP 98, BS 78) were also individually well-supported in the COI results (Fig. 3.17 b), while only the South African lineage was well-supported in the ITS2 ML results (Fig. 3.17 c; BS 70). However, the internal topology of the sampled individuals was not associated with their sampling localities or any observed morphological variation. Finally, *Cx*. (*Cux*.) *theileri* was only represented by a single sampled specimen in the 28S results, where it shared a clade with *Cx*. (*Cux*.) *univittatus* (Fig. 3.17 d).

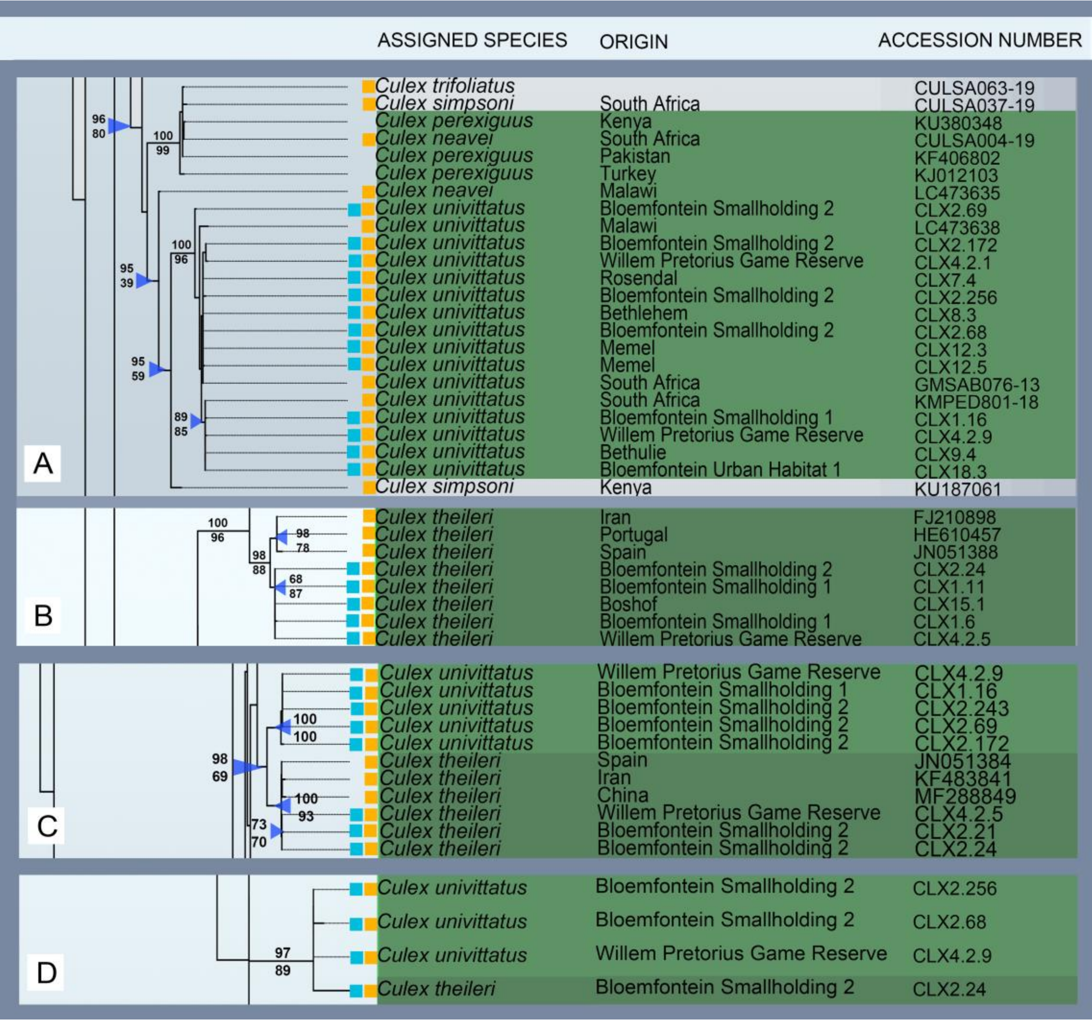


Fig. 3.17. The relationships of *Cx*. (*Cux*.) *univittatus* and *Cx*. (*Cux*.) *theileri* based on the maximum likelihood analyses: a, b) COI; c) ITS2; d) 28S. Figures represent the relevant subsets of Fig. 3.14, 3.15 and 3.16. Numbers represent significant support values (PP ≥ 95 or BS ≥ 70) for clades recovered in both the Bayesian (PP; top value) and maximum likelihood (BS; bottom value) analyses.

The remaining sampled species of the subgenus were members of the Pipiens Subgroup, which consisted of *Cx.* (*Cux.*) *quinquefasciatus* and *Cx.* (*Cux.*) *pipiens*. Here, the identities of all sampled specimens were corroborated by the results from the DNA sequence identifications. Both species were polyphyletic in the COI analyses (Fig. 3.18 a), where sampled *Cx.* (*Cux.*) *pipiens* specimens (CLX2.280, CLX2.82, CLX2.90, CLX5.2.7, CLX12.10, CLX21.10, CLX22.42) formed a well-supported clade distinct from all other members of the subgroup (PP 100, BS 97). The results recovered little phylogenetic distinction between the sampled *Cx.* (*Cux.*) *quinquefasciatus* specimens (CLX13.5, CLX18.2, CLX19.2, CLX21.5, CLX4.2.19) and all non-South African members of the Pipiens Subgroup. The subgroup's internal topology in the COI results was generally unresolved, except for the clades consisting of two sampled *Cx.* (*Cux.*) *quinquefasciatus* specimens (CLX21.5 + CLX13.5) and three sampled *Cx.* (*Cux.*) *pipiens* specimens (CLX22.42 + CLX2.82 + CLX12.10). Many of the sampled Pipiens Subgroup specimens displayed instances of morphological variation (Fig. 3.6 g–j), however, no unifying characteristics were present within the phylogenetic groupings. The placement of South African specimens was also not associated with their sampling locations.

The ITS2 datasets consisted of three publicly available *Cx.* (*Cux.*) *quinquefasciatus* sequences and four *Cx.* (*Cux.*) *pipiens* sequences (Fig. 3.18 b). The two sampled *Cx.* (*Cux.*) *pipiens* specimens (CLX2.280, CLX21.10) displayed no morphological variation, and the species as a whole formed a well-supported monophyletic grouping (PP 95, BS 93) with no consistent internal groupings. Conversely, *Cx.* (*Cux.*) *quinquefasciatus* was once again polyphyletic, where the Brazilian sequence grouped distantly from the Asian specimens. Lastly, both species were once again non-monophyletic in the 28S results, where *Cx.* (*Cux.*) *quinquefasciatus* was represented by three non-sampled South African sequences and two non-sampled *Cx.* (*Cux.*) *pipiens* specimens. These specimens were either included within a polytomy or intermixed with one another, with no correlation between their topology and geography (Fig. 3.18 c).

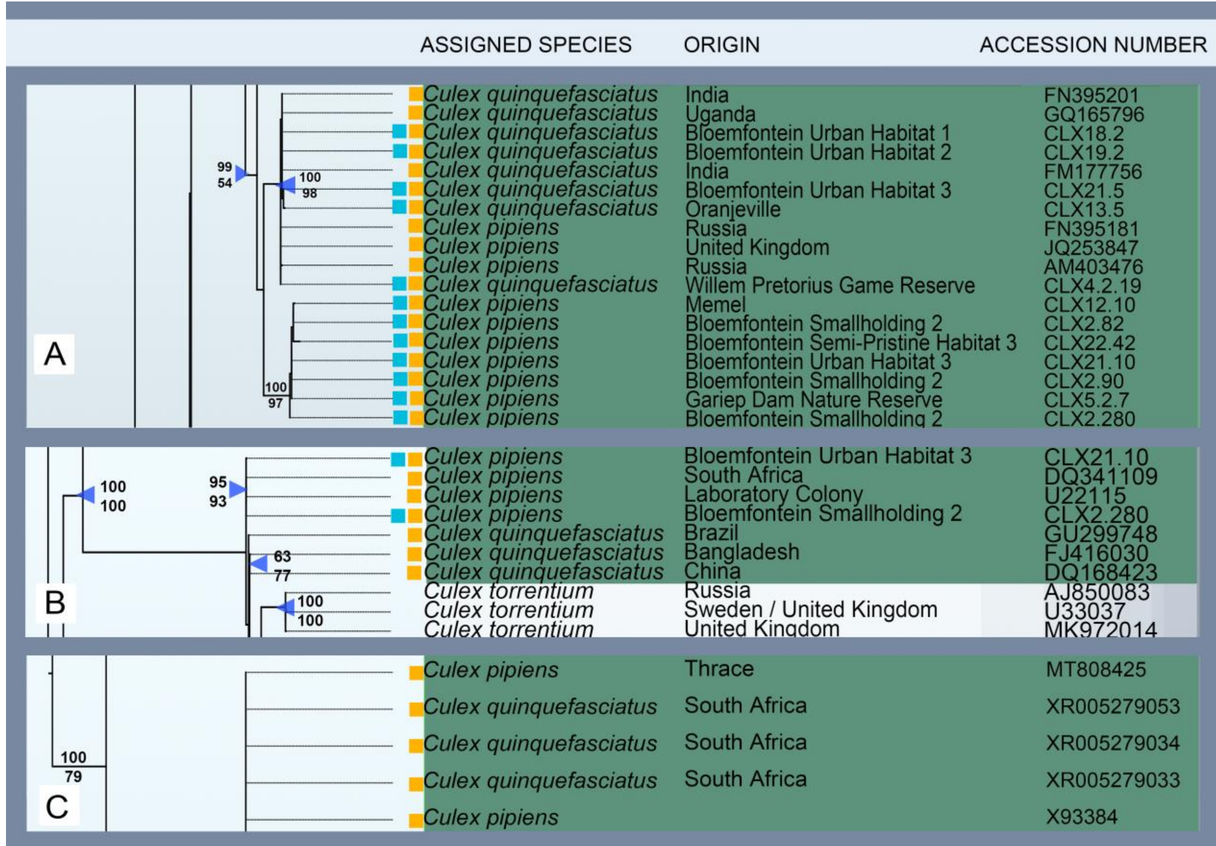


Fig. 3.18. The relationships of the *Cx.* (*Cux.*) *pipiens* and *Cx.* (*Cux.*) *quinquefasciatus* based on the maximum likelihood analyses: a) COI; b) ITS2; c) 28S. Figures represent the relevant subsets of Fig. 3.14, 3.15 and 3.16. Numbers represent significant support values (PP ≥ 95 or BS ≥ 70) for clades recovered in both the Bayesian (PP; top value) and maximum likelihood (BS; bottom value) analyses.

Subgenus *Melanoconion*

Melanoconion was represented by a total of 11 species in the COI and ITS2 datasets. The subgenus was paraphyletic with the inclusion of *Microculex* in the COI results (Fig. 3.14; PP 94) and monophyletic in the ITS2 results (Fig. 3.15; PP 99, BS 78). *Microculex* had a moderately to poorly supported sister relationship with the Pilosus Group in the COI results (PP 88, BS 32) and this subgenus' exclusion from the ITS2 dataset likely ensured the monophyly of *Melanoconion* in the accompanying results.

Melanoconion consisted of the Melanoconion and Spissipes Sections, where the topology was inconsistent between analyses. Both sections were polyphyletic and intermixed in the COI analyses, while the Spissipes Section was paraphyletic with the inclusion of a monophyletic Melanoconion Section (PP 95) in the ITS2 results. In the COI results, one coherent clade was recovered where the species were associated with their assigned sections. This clade consisted of several species from the Melanoconion Section: *Cx.* (*Mel.*) *aliciae* + *Cx.* (*Mel.*) *iolambdis* + *Cx.* (*Mel.*) *erraticus* + *Cx.* (*Mel.*) *atratus* + *Cx.* (*Mel.*) *ybarmis* (PP 99, BS 50).

Despite the differing topologies, several associations remained consistent between the DNA regions. This included the relatively close association between the Conspirator (*Cx. aliciae*) and Bastagarius

(*Cx. iolambdis*) Groups, with the addition of the Intrincatus Group in the ITS2 results. Here, both the Conspirator + Bastagarius (COI; PP 99, BS 92) and Conspirator + Intrincatus + Bastagarius Groups (ITS2; PP 96, BS 81) were well-supported. Both ML analyses also recovered the unsupported affiliation between the Crybda (*Cx. adamesi*) and Taeniopus (*Cx. cedecei*) Groups, with the addition of the Vomerifer Group in the ITS2 results.

Subgenus *Oculeomyia*

Oculeomyia was represented by three South African species, namely *Cx.* (*Ocu.*) *poicilipes*, *Cx.* (*Ocu.*) *bitaeniorhynchus* and *Cx.* (*Ocu.*) *annulioris*. In the COI analyses, *Oculeomyia* was paraphyletic with the inclusion of *Lophoceraomyia* (Fig. 3.14). Here, the internal topology was unsupported, and the *Oculeomyia* + *Lophoceraomyia* grouping was incorporated within the overarching subgenus *Culex* clade. In the ITS2 analyses, *Oculeomyia* was only represented by *Cx.* (*Ocu.*) *bitaeniorhynchus*, where it was similarly included within the larger *Culex* clade (Fig. 3.15). Both DNA regions recovered *Oculeomyia*'s close placement to *Culex*'s Sitiens Group, which was also unsupported. Despite the inclusion of *Lophoceraomyia* in the ITS2 dataset, it was not associated with *Oculeomyia*, as in the COI results.

3.7.2.2 *CULEX* SUBGENERA WITH A LIMITED REPRESENTATION

Several *Culex* subgenera were represented by a single DNA region within the public databases and could only be included within a single dataset. Several subgenera were also only represented by a single species, and thus inferences regarding their topology were limited. Since these taxa were often underrepresented within the literature, representatives were added to the current datasets to gain some insight into their phylogenetic history.

Here, both *Microculex* and *Phenacomyia* had limited coverage in the current analyses, since these subgenera each consisted of a single species within the COI dataset. *Microculex* was represented by a member of the Imitator Series, *Cx.* (*Mcx.*) *imitator*, and was included within the overarching *Melanoconion* clade as a sister taxon to *Melanoconion's* Pilosus Group (Fig. 3.14; PP 88). The second subgenus, *Phenacomyia*, was represented by *Cx.* (*Phc.*) *corniger* and was incorporated into the overarching subgenus *Culex* clade (Fig. 3.14), where its affiliations differed between the phylogenetic methods. Since the subgenera were not represented by the other DNA regions, their affiliations could not be substantiated.

A few other subgenera (*Barraudius*, *Lophoceraomyia* and *Neoculex*) were similarly represented by a single species, yet were included within multiple datasets. Here, *Cx.* (*Bar.*) *modestus* was added as a representative of *Barraudius* for all three DNA regions, where it was consistently incorporated within the overarching subgenus *Culex* clade (Fig. 3.14, 3.15 & 3.16). However, the specific affiliations of *Barraudius* were either unresolved or inconsistent between analyses.

The second subgenus*, Lophoceraomyia*, was represented by *Cx.* (*Lop.*) *cylindricus* in the COI and ITS2 datasets. This subgenus was included within an unsupported *Oculeomyia* clade in both COI analyses (Fig. 3.14), which was incorporated into the larger subgenus *Culex* clade. *Lophoceraomyia* was once again affiliated with the subgenus *Culex* in the ITS2 results, although its placement was inconsistent between the phylogenetic methods. Here, the subgenus was placed basally to a clade consisting of *Culex* + *Barraudius* + *Oculeomyia* in the Bayesian results, and formed an unsupported ML grouping with *Barraudius* within the overarching *Culex* clade (Fig. 3.15).

The third subgenus, *Neoculex* was represented by *Cx.* (*Ncx.*) *impudicus* in the COI and 28S datasets. Both COI analyses recovered a clade consisting of *Neoculex* + *Eumelanomyia* + *Maillotia* (Fig. 3.14), where the subgenera were either included in a shared polytomy or an unresolved clade. In the 28S results, *Neoculex* was situated basally to all remaining members of the genus (Fig. 3.16). Since *Eumelanomyia* and *Maillotia* were not included in the 28S dataset, their affiliations with *Neoculex* could not be confirmed.

Three other subgenera were represented by multiple species within a single dataset. This included *Culiciomyia*, *Eumelanomyia* and *Maillotia*, which were all included in the COI analyses. Three *Culiciomyia* species were represented in the dataset, including two South African species (*Cx. nebulosus* and *Cx. cinereus*). *Culiciomyia* formed a moderately to well-supported monophyletic clade in the COI results (PP 99, BS 65), which was incorporated within the larger subgenus *Culex* clade (Fig. 3.14). The subgenus' Nebulosus Group was also monophyletic and well-supported (PP 100, BS 100), yet there was little phylogenetic distinction between its two members (*Cx. nebulosus* and *Cx. cinereus*) (Fig. 3.19). All East African Nebulosus Group specimens were grouped together in the ML results, while the geographically distant Malawian specimen was positioned basally.

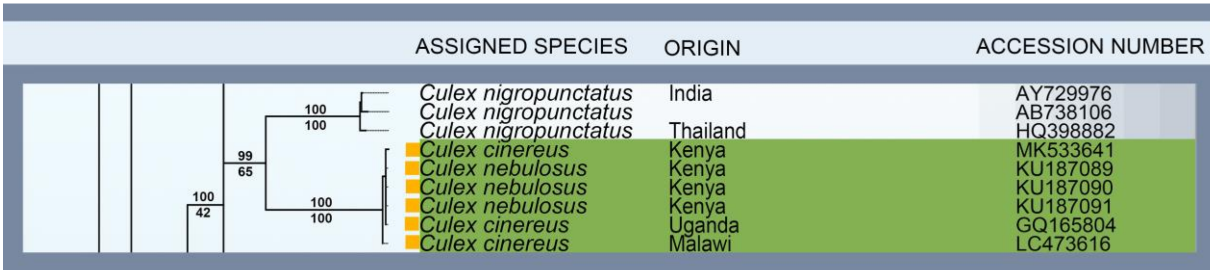


Fig. 3.19. The relationships of the Nebulosus Group based on the COI maximum likelihood analyses. The figure represents the relevant subset of Fig. 3.14. Numbers represent significant support values (PP ≥ 95 or BS ≥ 70) for clades recovered in both the Bayesian (PP; top value) and maximum likelihood (BS; bottom value) analyses.

The two remaining subgenera, *Eumelanomyia* and *Maillotia* shared a clade in the COI results. Three *Eumelanomyia* species were added to the dataset, including *Cx.* (*Eum.*) *rima* as a South African species. *Maillotia* was represented by two species, including one sampled individual of *Cx.* (*Mai.*) *salisburiensis* (CLX9.2). Since *Cx.* (*Mai.*) *salisburiensis* was not represented within the GenBank database, its morphological identity could not be substantiated by DNA comparisons. However, the BLAST results nonetheless produced a relatively close match to another species of the same subgenus.

The weak phylogenetic resolution produced polytomous clades in the Bayesian results (results not shown) and likely contributed to the polyphyletic arrangement of the subgenera in the ML analysis (Fig. 3.14). The Mochthogenes Group of *Eumelanomyia* was also polyphyletic in the ML analysis, where its subgroups (Tenuipalpis & Foliatus) were each weakly associated with a distinct *Maillotia* clade. Here, both subgenera were divided into two unsupported groupings, consisting of *Cx.* (*Mai.*) *salisburiensis* (Seyrigi Group) + *Eumelanomyia* (Tenuipalpis Subgroup) + *Cx.* (*Eum.*) *rima* (Rima Subgroup) and *Maillotia* (Hortensis Group) + *Eumelanomyia* (Foliatus Subgroup). Since these subgenera were only represented in the COI dataset and its structure was poorly resolved, its affiliations could not be confirmed.

3.7.3 *ANOPHELES*

The *Anopheles* datasets consisted of comparatively few subgenera and many taxonomic subdivisions. Here, the genus was represented by five subgenera, five sections, 14 series and 33 groups, including 65 species and 385 sequences. The datasets incorporated 19 South African species and four sequences originating from sampled *An.* (*Cel.*) *cydippis* / *squamosus* specimens. The identity of the sampled specimens could not be verified through sequence comparisons. However, the key provided by Gillies and Coetzee (1987) unambiguously identified sampled specimens as members of the Squamosus Group (*An. cydippis* or *squamosus*).

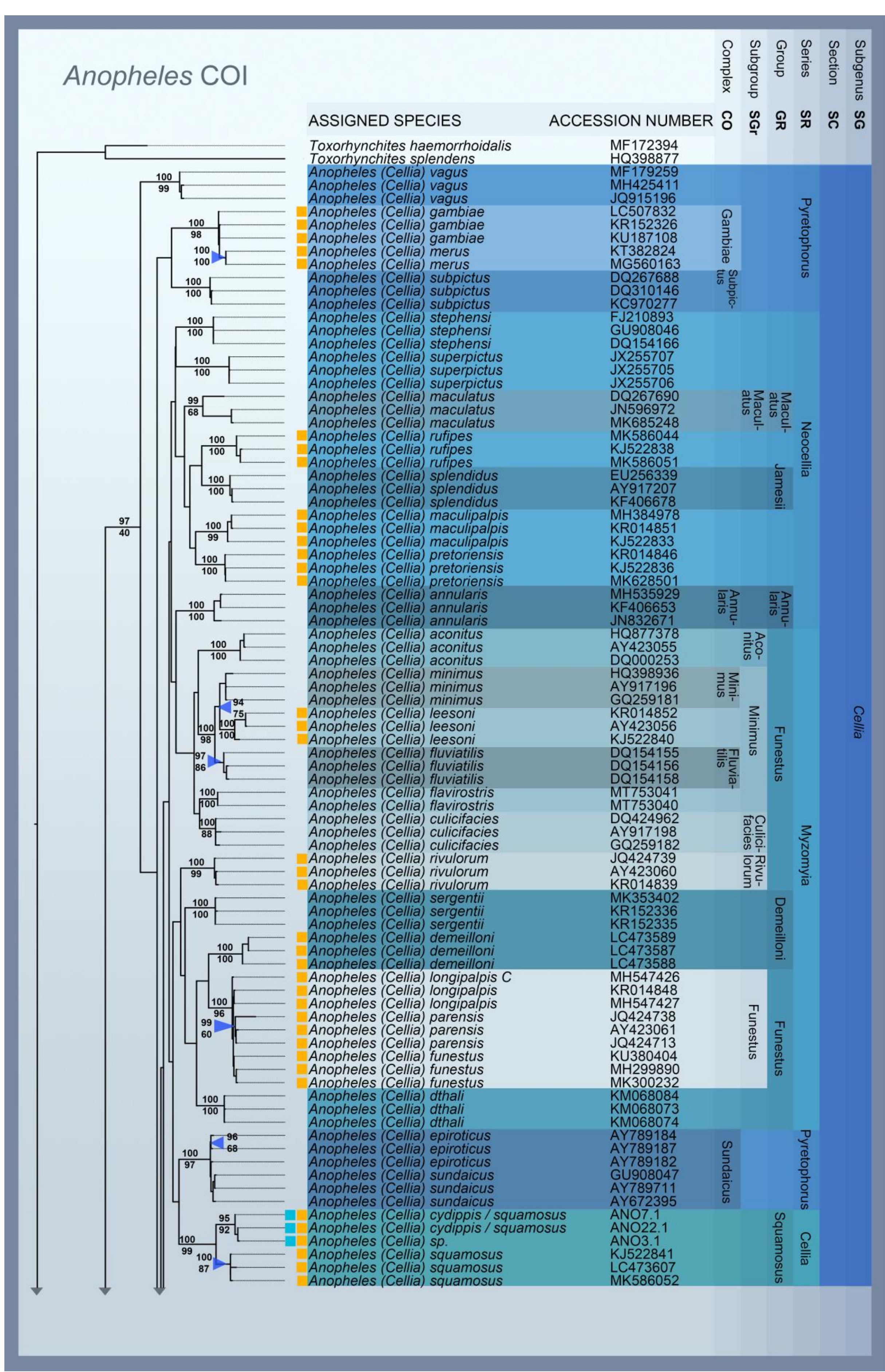
The COI dataset yielded the greatest taxonomic coverage, where it represented five subgenera and a total of 59 species. The alignment consisted of 658 sites, including 378 conserved, 280 variable and 247 parsimony informative positions. The internal relationships were generally very weakly resolved, producing either a large Bayesian polytomy or weakly supported internal relationships in the ML analysis (Fig. 3.20). However, the resolution of terminal groupings was greater, where multiple clades were well-supported and shared between the phylogenetic methods. Two of the subgenera were represented by a single species, where they were often closely associated with the non-monophyletic larger taxa.

The ITS2 dataset was slightly smaller and consisted of four subgenera and 63 species. The ITS2 alignment consisted of 2,113 sites, including 608 conserved, 1,389 variable and 1,218 parsimony informative positions. The internal resolution was slightly improved in the ITS2 results and numerous terminal relationships were well-supported (Fig. 3.21). Many of these well-supported clades also consisted of relatively large species groupings that remained consistent between the phylogenetic methods. One subgenus was represented by a single species, while the larger subgenera were once again recovered as non-monophyletic.

Finally, the 28S dataset was substantially smaller and represented three subgenera and 14 species. The alignment consisted of 465 sites, including 312 conserved, 148 variable and 134 parsimony informative sites. The internal relationships had a reasonable degree of support, while terminal

relationships were intermittently unresolved (Fig. 3.22). Nonetheless, portions of the topology remained consistent between the phylogenetic methods, where the largest subgenus was monophyletic, while the remaining two subgenera clustered together.

Numerous findings were supported by multiple DNA regions, where many of the taxonomic subdivisions were recovered as poly- or paraphyletic assemblages. This included the non-monophyly of *Anopheles* and *Nyssorhynchus*, the polyphyly of four associated sections (Angusticorn, Laticorn, Argyritarsis and Albimanus), the non-monophyly of six series (Anopheles, Myzorhynchus, Argyritarsis, Albitarsis, Oswaldoi and Pyretophorus) and the polyphyly of three species groups (Barbirostris, Demeilloni and Funestus).



Anopheles COI
ASSIGNED SPECIES
ACCESSION NUMBER
CO Complex
SGr Subgroup
GR Group
SR Series
SC Section
SG Subgenus
Toxorhynchites haemorrhoidalis MF172394
Toxorhynchites splendens HQ398877
Anopheles (Cellia) vagus MF179259
Anopheles (Cellia) vagus MH425411
Anopheles (Cellia) vagus JQ915196
Anopheles (Cellia) gambiae LC507832
Anopheles (Cellia) gambiae KR152326
Anopheles (Cellia) gambiae KU187108
Anopheles (Cellia) merus KT382824
Anopheles (Cellia) merus MG560163
Anopheles (Cellia) subpictus DQ267688
Anopheles (Cellia) subpictus DQ310146
Anopheles (Cellia) subpictus KC970277
Anopheles (Cellia) stephensi FJ210893
Anopheles (Cellia) stephensi GU908046
Anopheles (Cellia) stephensi DQ154166
Anopheles (Cellia) superpictus JX255707
Anopheles (Cellia) superpictus JX255705
Anopheles (Cellia) superpictus JX255706
Anopheles (Cellia) maculatus DQ267690
Anopheles (Cellia) maculatus JN596972
Anopheles (Cellia) maculatus MK685248
Anopheles (Cellia) rufipes MK586044
Anopheles (Cellia) rufipes KJ522838
Anopheles (Cellia) rufipes MK586051
Anopheles (Cellia) splendidus EU256339
Anopheles (Cellia) splendidus AY917207
Anopheles (Cellia) splendidus KF406678
Anopheles (Cellia) maculipalpis MH384978
Anopheles (Cellia) maculipalpis KR014851
Anopheles (Cellia) maculipalpis KJ522833
Anopheles (Cellia) pretoriensis KR014846
Anopheles (Cellia) pretoriensis KJ522836
Anopheles (Cellia) pretoriensis MK628501
Anopheles (Cellia) annularis MH535929
Anopheles (Cellia) annularis KF406653
Anopheles (Cellia) annularis JN832671
Anopheles (Cellia) aconitus HQ877378
Anopheles (Cellia) aconitus AY423055
Anopheles (Cellia) aconitus DQ000253
Anopheles (Cellia) minimus HQ398936
Anopheles (Cellia) minimus AY917196
Anopheles (Cellia) minimus GQ259181
Anopheles (Cellia) leesoni KR014852
Anopheles (Cellia) leesoni AY423056
Anopheles (Cellia) leesoni KJ522840
Anopheles (Cellia) fluviatilis DQ154155
Anopheles (Cellia) fluviatilis DQ154156
Anopheles (Cellia) fluviatilis DQ154158
Anopheles (Cellia) flavirostris MT753041
Anopheles (Cellia) flavirostris MT753040
Anopheles (Cellia) culicifacies DQ424962
Anopheles (Cellia) culicifacies AY917198
Anopheles (Cellia) culicifacies GQ259182
Anopheles (Cellia) rivulorum JQ424739
Anopheles (Cellia) rivulorum AY423060
Anopheles (Cellia) rivulorum KR014839
Anopheles (Cellia) sergentii MK353402
Anopheles (Cellia) sergentii KR152336
Anopheles (Cellia) sergentii KR152335
Anopheles (Cellia) demeilloni LC473589
Anopheles (Cellia) demeilloni LC473587
Anopheles (Cellia) demeilloni LC473588
Anopheles (Cellia) longipalpis C MH547426
Anopheles (Cellia) longipalpis KR014848
Anopheles (Cellia) longipalpis MH547427
Anopheles (Cellia) parensis JQ424738
Anopheles (Cellia) parensis AY423061
Anopheles (Cellia) parensis JQ424713
Anopheles (Cellia) funestus KU380404
Anopheles (Cellia) funestus MH299890
Anopheles (Cellia) funestus MK300232
Anopheles (Cellia) dthali KM068084
Anopheles (Cellia) dthali KM068073
Anopheles (Cellia) dthali KM068074
Anopheles (Cellia) epiroticus AY789184
Anopheles (Cellia) epiroticus AY789187
Anopheles (Cellia) epiroticus AY789182
Anopheles (Cellia) sundaicus GU908047
Anopheles (Cellia) sundaicus AY789711
Anopheles (Cellia) sundaicus AY672395
Anopheles (Cellia) cydippis / squamosus ANO7.1
Anopheles (Cellia) cydippis / squamosus ANO22.1
Anopheles (Cellia) sp. ANO3.1
Anopheles (Cellia) squamosus KJ522841
Anopheles (Cellia) squamosus LC473607
Anopheles (Cellia) squamosus MK586052
Gambiae
Subpictus
Maculatus
Maculatus
Jamesii
Annularis
Annularis
Aconitus
Minimus
Minimus
Fluviatilis
Culicifacies
Rivulorum
Funestus
Demeilloni
Funestus
Funestus
Sundaicus
Squamosus
Pyretophorus
Neocellia
Myzomyia
Pyretophorus
Cellia
Cellia

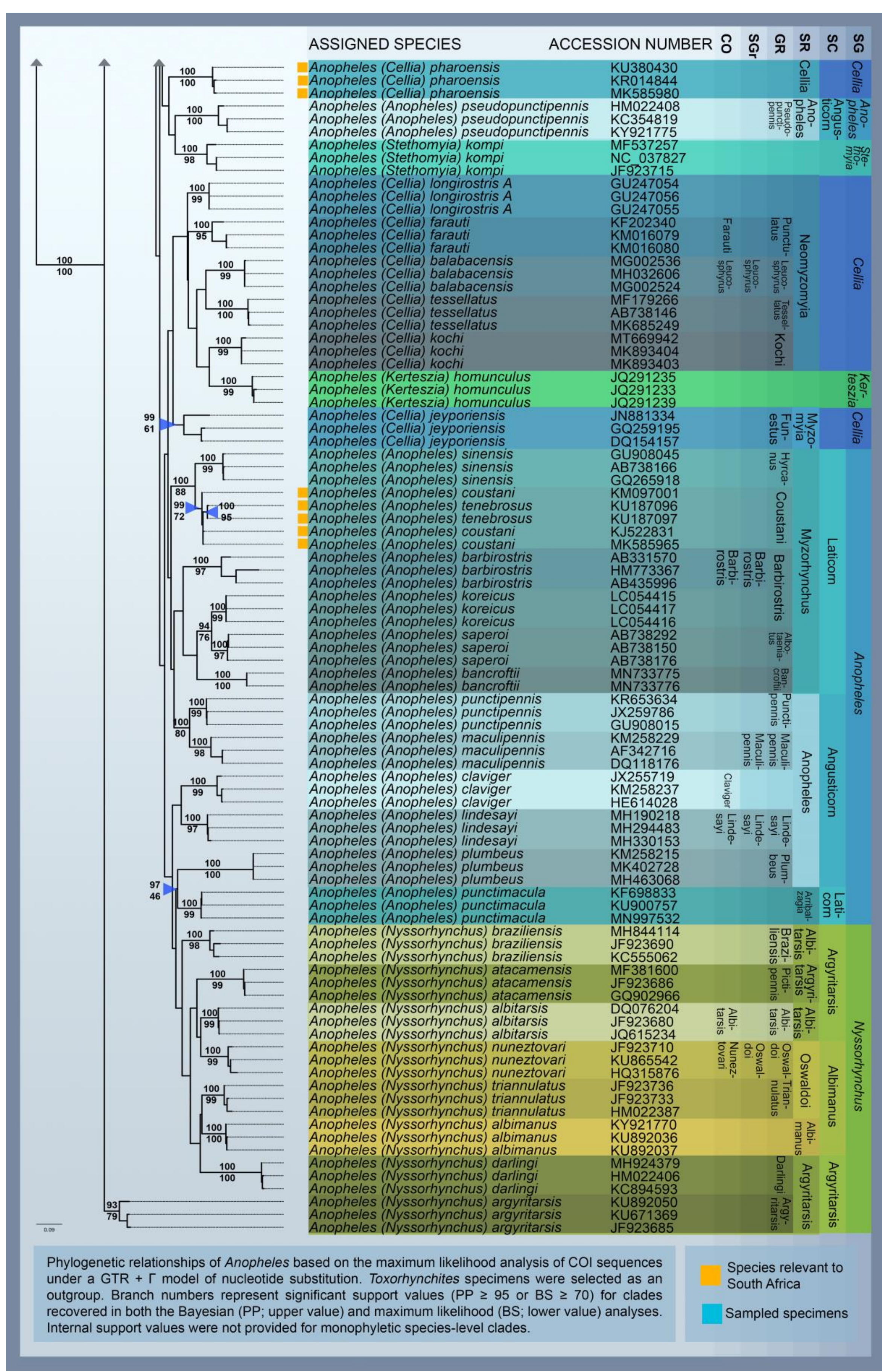
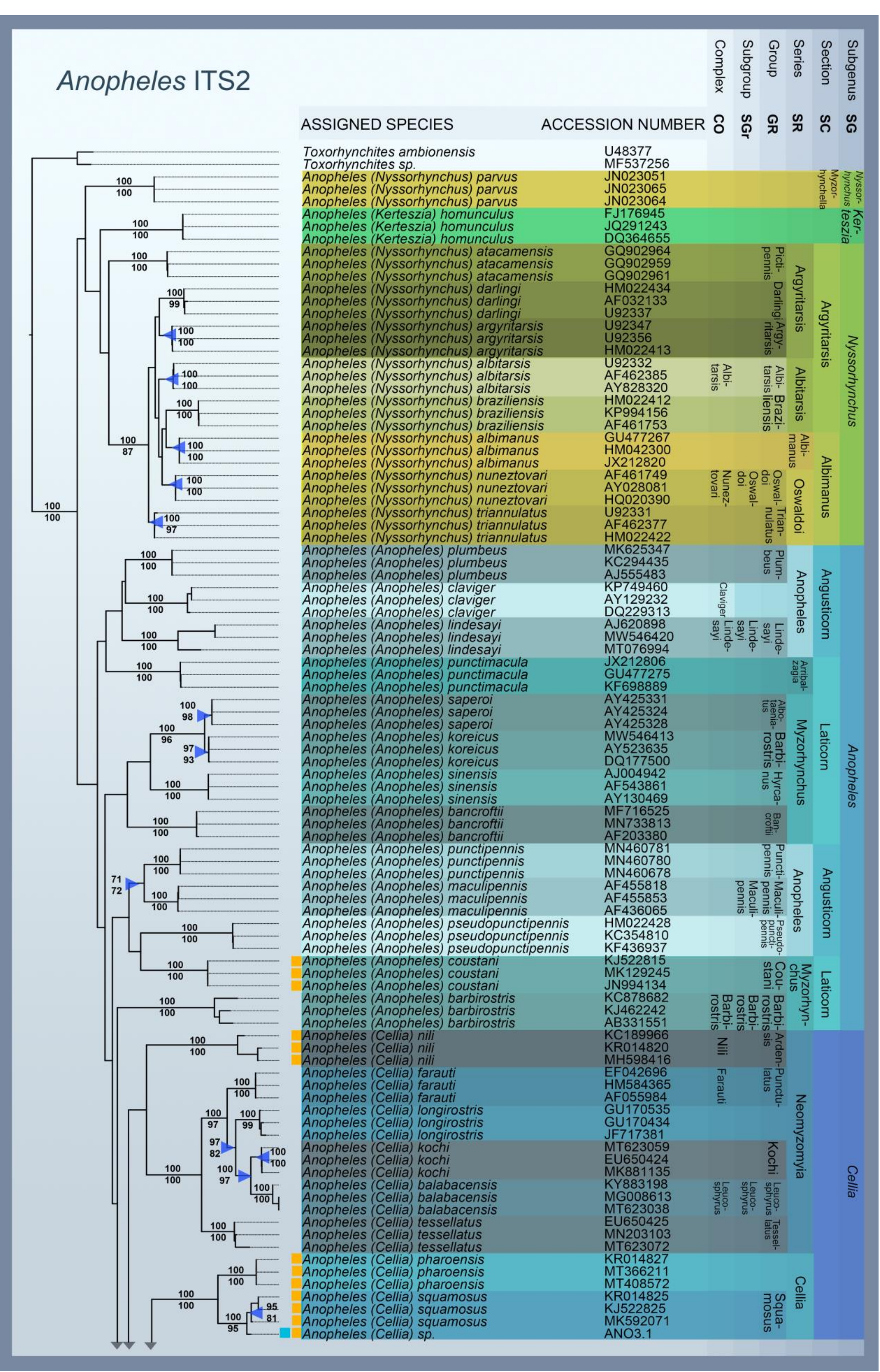


Fig. 3.20. Phylogenetic relationships of *Anopheles* based on the COI nucleotide sequences.



Anopheles ITS2
ASSIGNED SPECIES
ACCESSION NUMBER
CO Complex
SGr Subgroup
GR Group
SR Series
SC Section
SG Subgenus
Toxorhynchites ambionensis U48377
Toxorhynchites sp. MF537256
Anopheles (Nyssorhynchus) parvus JN023051
Anopheles (Nyssorhynchus) parvus JN023065
Anopheles (Nyssorhynchus) parvus JN023064
Anopheles (Kerteszia) homunculus FJ176945
Anopheles (Kerteszia) homunculus JQ291243
Anopheles (Kerteszia) homunculus DQ364655
Anopheles (Nyssorhynchus) atacamensis GQ902964
Anopheles (Nyssorhynchus) atacamensis GQ902959
Anopheles (Nyssorhynchus) atacamensis GQ902961
Anopheles (Nyssorhynchus) darlingi HM022434
Anopheles (Nyssorhynchus) darlingi AF032133
Anopheles (Nyssorhynchus) darlingi U92337
Anopheles (Nyssorhynchus) argyritarsis U92347
Anopheles (Nyssorhynchus) argyritarsis U92356
Anopheles (Nyssorhynchus) argyritarsis HM022413
Anopheles (Nyssorhynchus) albitarsis U92332
Anopheles (Nyssorhynchus) albitarsis AF462385
Anopheles (Nyssorhynchus) albitarsis AY828320
Anopheles (Nyssorhynchus) braziliensis HM022412
Anopheles (Nyssorhynchus) braziliensis KP994156
Anopheles (Nyssorhynchus) braziliensis AF461753
Anopheles (Nyssorhynchus) albimanus GU477267
Anopheles (Nyssorhynchus) albimanus HM042300
Anopheles (Nyssorhynchus) albimanus JX212820
Anopheles (Nyssorhynchus) nuneztovari AF461749
Anopheles (Nyssorhynchus) nuneztovari AY028081
Anopheles (Nyssorhynchus) nuneztovari HQ020390
Anopheles (Nyssorhynchus) triannulatus U92331
Anopheles (Nyssorhynchus) triannulatus AF462377
Anopheles (Nyssorhynchus) triannulatus HM022422
Anopheles (Anopheles) plumbeus MK625347
Anopheles (Anopheles) plumbeus KC294435
Anopheles (Anopheles) plumbeus AJ555483
Anopheles (Anopheles) claviger KP749460
Anopheles (Anopheles) claviger AY129232
Anopheles (Anopheles) claviger DQ229313
Anopheles (Anopheles) lindesayi AJ620898
Anopheles (Anopheles) lindesayi MW546420
Anopheles (Anopheles) lindesayi MT076994
Anopheles (Anopheles) punctimacula JX212806
Anopheles (Anopheles) punctimacula GU477275
Anopheles (Anopheles) punctimacula KF698889
Anopheles (Anopheles) saperoi AY425331
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Anopheles (Anopheles) saperoi AY425328
Anopheles (Anopheles) koreicus MW546413
Anopheles (Anopheles) koreicus AY523635
Anopheles (Anopheles) koreicus DQ177500
Anopheles (Anopheles) sinensis AJ004942
Anopheles (Anopheles) sinensis AF543861
Anopheles (Anopheles) sinensis AY130469
Anopheles (Anopheles) bancroftii MF716525
Anopheles (Anopheles) bancroftii MN733813
Anopheles (Anopheles) bancroftii AF203380
Anopheles (Anopheles) punctipennis MN460781
Anopheles (Anopheles) punctipennis MN460780
Anopheles (Anopheles) punctipennis MN460678
Anopheles (Anopheles) maculipennis AF455818
Anopheles (Anopheles) maculipennis AF455853
Anopheles (Anopheles) maculipennis AF436065
Anopheles (Anopheles) pseudopunctipennis HM022428
Anopheles (Anopheles) pseudopunctipennis KC354810
Anopheles (Anopheles) pseudopunctipennis KF436937
Anopheles (Anopheles) coustani KJ522815
Anopheles (Anopheles) coustani MK129245
Anopheles (Anopheles) coustani JN994134
Anopheles (Anopheles) barbirostris KC878682
Anopheles (Anopheles) barbirostris KJ462242
Anopheles (Anopheles) barbirostris AB331551
Anopheles (Cellia) nili KC189966
Anopheles (Cellia) nili KR014820
Anopheles (Cellia) nili MH598416
Anopheles (Cellia) farauti EF042696
Anopheles (Cellia) farauti HM584365
Anopheles (Cellia) farauti AF055984
Anopheles (Cellia) longirostris GU170535
Anopheles (Cellia) longirostris GU170434
Anopheles (Cellia) longirostris JF717381
Anopheles (Cellia) kochi MT623059
Anopheles (Cellia) kochi EU650424
Anopheles (Cellia) kochi MK881135
Anopheles (Cellia) balabacensis KY883198
Anopheles (Cellia) balabacensis MG008613
Anopheles (Cellia) balabacensis MT623038
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Anopheles (Cellia) tessellatus MN203103
Anopheles (Cellia) tessellatus MT623072
Anopheles (Cellia) pharoensis KR014827
Anopheles (Cellia) pharoensis MT366211
Anopheles (Cellia) pharoensis MT408572
Anopheles (Cellia) squamosus KR014825
Anopheles (Cellia) squamosus KJ522825
Anopheles (Cellia) squamosus MK592071
Anopheles (Cellia) sp. ANO3.1
Nyssorhynchus
Kerteszia
Anopheles
Cellia
Myzorhynchella
Argyritarsis
Albimanus
Angusticorn
Laticorn
Neomyzomyia
Myzorhynchus
Anopheles
Arribalzagia
Albitarsis
Oswaldoi
Albimanus
Pictipennis
Darlingi
Argyritarsis
Albitarsis
Braziliensis
Oswaldoi
Triannulatus
Nuneztovari
Plumbeus
Claviger
Lindesayi
Albotaeniatus
Barbirostris
Hyrcanus
Bancroftii
Punctipennis
Maculipennis
Pseudopunctipennis
Coustani
Arden-
Punctulatus
Nili
Farauti
Kochi
Leucosphyrus
Tessellatus
Cellia
Squamosus

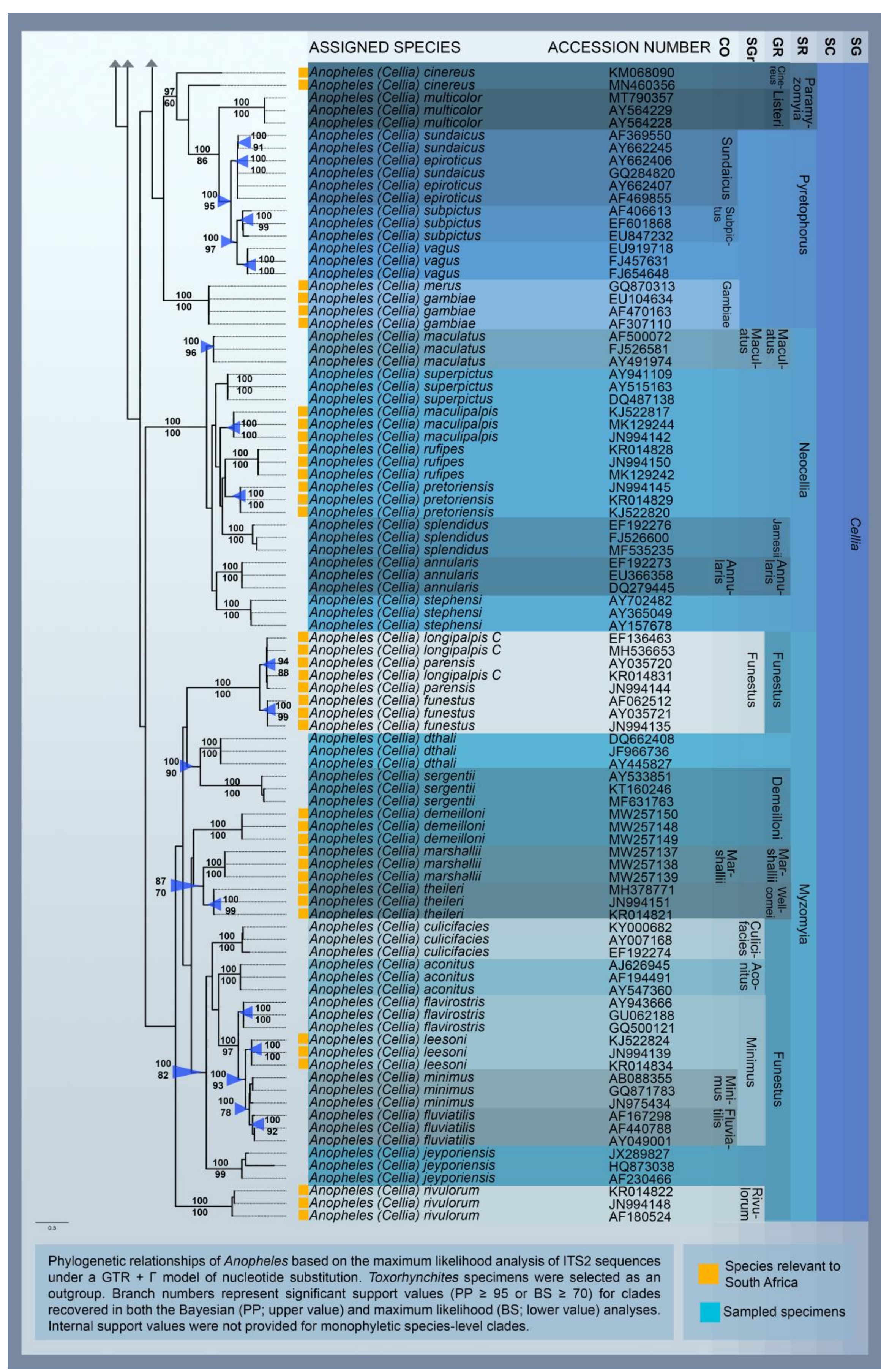

Fig. 3.21. Phylogenetic relationships of *Anopheles* based on the ITS2 nucleotide sequences.

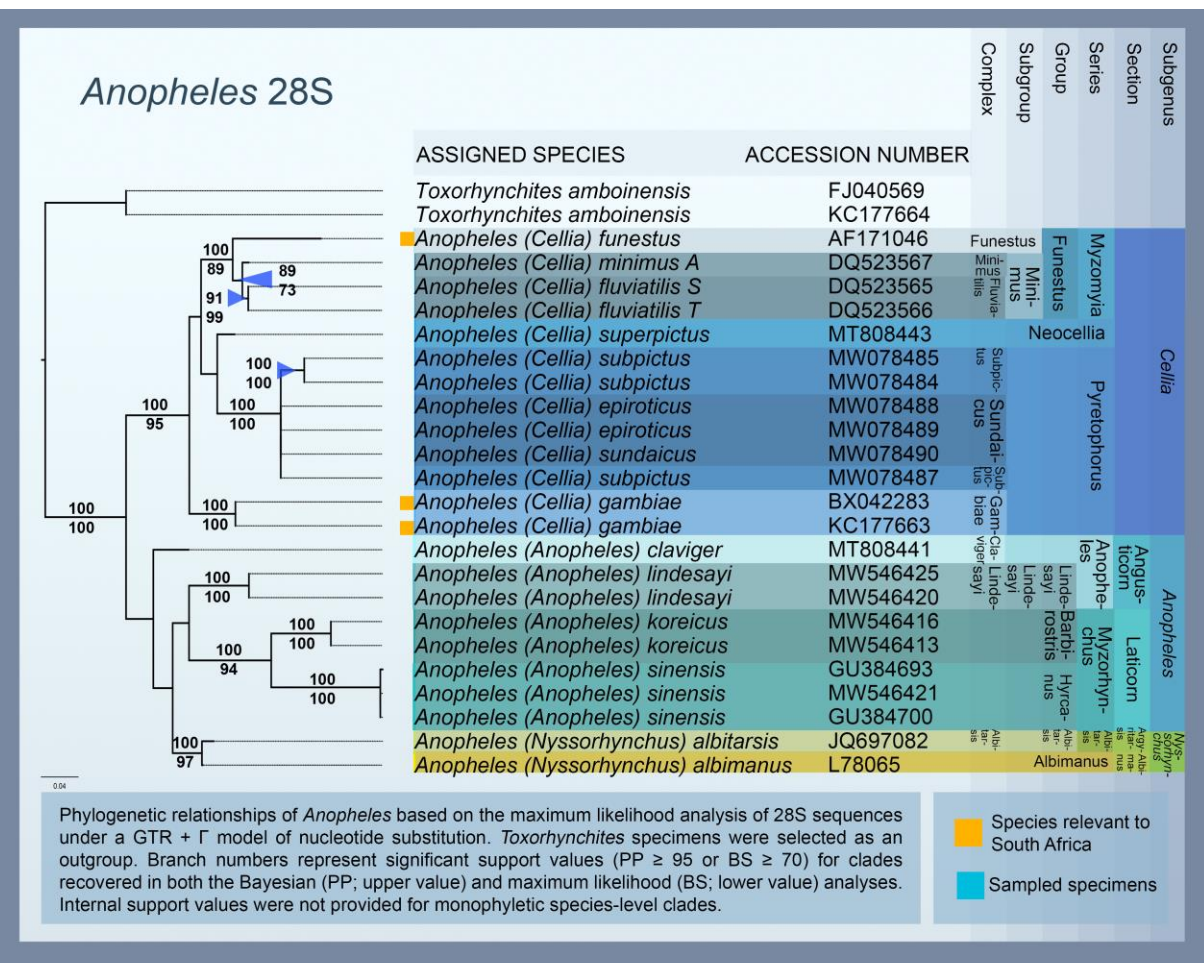

Fig. 3.**22**. Phylogenetic relationships of *Anopheles* based on the 28S nucleotide sequences.

3.7.3.1 *ANOPHELES* SUBGENERA WITH A COMPREHENSIVE REPRESENTATION

Three *Anopheles* subgenera, namely *Anopheles*, *Cellia* and *Nyssorhynchus*, were included in all three datasets and represented by a relatively large number of species. This extensive taxonomic coverage provided valuable insights into the relevant taxa's internal structure, stability and affiliations. These results raised doubt about the validity of *Anopheles'* numerous formal subdivisions.

Subgenus *Anopheles*

The subgenus was represented by 14 species, including two Coustani Group members relevant to South Africa (*An. coustani* & *An. tenebrosus*). All three DNA regions recovered *Anopheles* as a non-monophyletic assemblage, while the topology differed between analyses. The three Bayesian analyses were weakly resolved and the subgenus was divided into multiple polytomous clades. The resolution was improved in the ML results, where *Anopheles* was polyphyletic in the COI results (Fig. 3.20), and paraphyletic with the inclusion of *Cellia* and *Nyssorhynchus* in the ITS2 and 28S results, respectively (Fig. 3.21 & 3.22). Another subgenus, *Stethomyia*, was similarly associated with the subgenus

Anopheles, yet it was only represented in the COI dataset. Here, the results produced an unsupported clade consisting of *Stethomyia* + *An.* (*Ano.*) *pseudopunctipennis*.

The non-monophyly of *Anopheles* also contributed to the polyphyly of two of its sections, namely the Angusticorn and Laticorn Sections. In most analyses, both sections consisted of numerous disjointed clades, where the polyphyletic clades were often intermixed in the COI and ITS2 results (Fig. 3.20 & 3.21). On the other hand, the Laticorn Section was monophyletic in the 28S results, where it was well-supported (Fig. 3.22; PP 100, BS 94). This monophyly was likely the result of the relatively low taxonomic coverage in the 28S dataset.

Two *Anopheles* series were similarly recovered as non-monophyletic. The Myzorhynchus Series was paraphyletic with the inclusion of the Anopheles Series (in part) (Maculipennis + Punctipennis Groups) in the COI analyses (Fig. 3.20), while the ITS2 results produced multiple polytomous or polyphyletic Myzorhynchus clades (Fig. 3.21). However, the series was only represented by two species in the 28S analyses, where it formed a well-supported monophyletic grouping (Fig. 3.22; PP 100, BS 94). One of the Myzorhynchus species groups, the Barbirostris Group, was recovered as polyphyletic in both the COI and ITS2 analyses, while the group was only represented by a single species in the 28S dataset. The Anopheles Series was also non-monophyletic in all analyses, either as a polyphyletic assemblage or spread across numerous polytomous clades. The final series, Arribalzagia, was represented by a single species (*An. punctimacula*) and its relationships were inconsistent and unsupported.

However, numerous associations were well-supported and recovered by multiple DNA regions. This included the Myzorhynchus Series clade consisting of the Albotaeniatus Group + *An.* (*Ano.*) *koreicus*, recovered in both the COI (Fig. 3.20; PP 94, BS 76) and ITS2 (Fig. 3.21; PP 100, BS 96) analyses. Here, *An.* (*Ano.*) *koreicus* also represented one of the polyphyletic Barbirostris Group clades, which was consistently affiliated with the Albotaeniatus Group, while the associations of the second Barbirostris Group clade (*An. barbirostris*) were inconsistent. *Anopheles* (*Ano.*) *koreicus* was also associated with the Hyrcanus Group, where both taxa shared a clade in the ITS2 results and were placed as sister taxa in the 28S results (Fig. 3.22; PP 100, BS 94). Furthermore, both the COI and ITS2 results recovered the close placement of the Lindesayi and Claviger Complexes, and the well-supported Maculipennis + Punctipennis Group clade (COI: PP 100, BS 80; ITS2: BS 72).

The Coustani Group included the only South African representatives of the subgenus *Anopheles* within the current datasets. In the COI results, the Coustani Group was monophyletic, while *An.* (*Ano.*) *coustani* was paraphyletic with the inclusion of *An.* (*Ano.*) *tenebrosus* (Fig. 3.23). The topology of *An.* (*Ano.*) *coustani* specimens was not associated with their geography, since the West African specimens (Guinea-Bissau & Mali) did not share a clade. Since the Coustani Group was only represented by *An.* (*Ano.*) *coustani* in the ITS2 analyses, its paraphyly could not be confirmed. Additional affiliations of the group were inconsistent, despite its well-supported relationship with the Hyrcanus Group in the COI results (PP 100, BS 88).

Fig. 3.23. The relationships of the Coustani Group based on the COI maximum likelihood analyses. The figure represents the relevant subset of Fig. 3.20. Numbers represent significant support values (PP ≥ 95 or BS ≥ 70) for clades recovered in both the Bayesian (PP; top value) and maximum likelihood (BS; bottom value) analyses.

Subgenus *Cellia*

Cellia was very well-represented within the *Anopheles* datasets and consisted of more than 40 species, including 17 species relevant to South Africa. Additionally, this subgenus was represented by a few sampled individuals of *An.* (*Cel.*) *cydippis* / *squamosus* (ANO3.1, ANO7.1, ANO22.1) in the COI and ITS2 datasets. The topology of *Cellia* was inconsistent between analyses, where it consisted of numerous polytomous clades in the COI and ITS2 Bayesian analyses and weakly supported polyphyletic clades in the COI ML results (Fig. 3.20). However, the subgenus was monophyletic in the ITS2 ML (Fig. 3.21) and 28S (Fig. 3.22; PP 100, BS 95) analyses, where the latter monophyly was likely due to the relatively low taxonomic coverage.

The monophyly of most series was inconsistent between the DNA regions, since the series tended to be polyphyletic in the COI analyses and monophyletic in the ITS2 and 28S results. The Cellia Series was represented by a few South African species in the COI and ITS2 datasets, which consisted of *An.* (*Cel.*) *squamosus*, *An.* (*Cel.*) *pharoensis* and sampled individuals of *An.* (*Cel.*) *cydippis* / *squamosus*. The COI results produced two monophyletic and well-supported lineages (Fig. 3.24 a), consisting of morphologically similar sampled *An.* (Cel.) *cydippis* / *squamosus* specimens (PP 95, BS 92) and non-sampled African *An.* (*Cel.*) *squamosus* specimens (PP 100, BS 87). The sampled specimens were stratified according to their sampling location, where the two Bloemfontein specimens (ANO3.1, ANO22.1) formed a single clade, while the Rosendal specimen (ANO7.1) was positioned basally. In the ITS2 analyses (Fig. 3.24 b), all non-sampled specimens once again formed a well-supported monophyletic grouping (PP 95, BS 81), which in turn was related to the single sampled specimen (ANO3.1). The combined Squamosus Group clade of sampled and non-sampled specimens was also well-supported for both DNA regions (COI: PP 100, BS 99; ITS2: 100, BS 95).

The placement of the final Cellia Series species, *An.* (*Cel.*) *pharoensis*, was inconsistent between the DNA regions. The species was sister to *An.* (*Cel.*) *squamosus* in the ITS2 analyses with a high degree of support (Fig. 3.21; PP 100, BS 100), forming a monophyletic Cellia Series clade. However, the series was polyphyletic in the COI results due to the distant placement of *An.* (*Cel.*) *pharoensis* (Fig. 3.20).

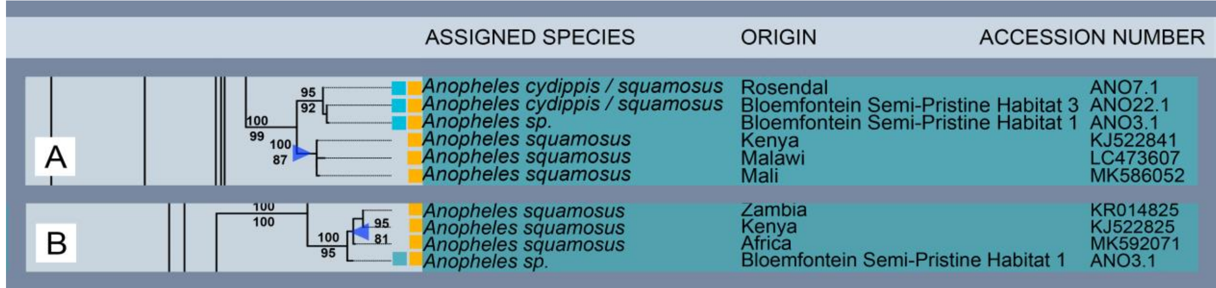

Fig. 3.24. The relationships of the Squamosus Group based on the maximum likelihood analyses: a) COI; b) ITS2. Figures represent the relevant subsets of Fig. 3.20 and 3.21. Numbers represent significant support values (PP ≥ 95 or BS ≥ 70) for clades recovered in both the Bayesian (PP; top value) and maximum likelihood (BS; bottom value) analyses.

The Pyretophorus Series yielded more consistent results, since it was polyphyletic in all analyses. The series consisted of discontinuous clades in the COI and 28S results (Fig. 3.20 & 3.22), while it was interrupted by another series in the ITS2 results (Fig. 3.21). Although the topology generally differed between analyses, two Pyretophorus groupings remained consistent between the multiple DNA regions. This included the well-supported monophyly of the Gambiae (COI: PP 100, BS 98; ITS2: PP 100, BS 100) and Sundaicus (COI: PP 100, BS 97; ITS2: PP 100, BS 86) Complexes in the COI and ITS2 analyses.

The Gambiae Complex was represented by two species, *An.* (*Cel.*) *gambiae* and *An.* (*Cel.*) *merus*, which both occur in South Africa. The phylogenetic resolution of these species was limited (Fig. 3.25), where the members shared a combined polytomy in the Bayesian ITS2 results, while *An.* (*Cel.*) *gambiae* was infrequently recovered as non-monophyletic. Here, *An.* (*Cel.*) *gambiae* specimens were not stratified according to their geography, since the West African specimens (Togo & Ghana; Benin & Gambia) did not group together in the COI and ITS2 results. However, *Anopheles* (*Cel.*) *gambiae* was the only representative of the complex in the 28S results.

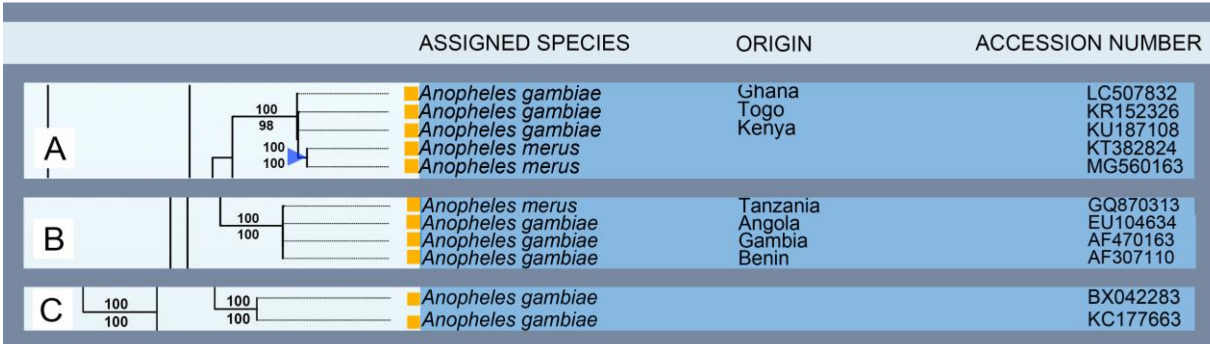

Fig. 3.25. The relationships of the Gambiae Complex based on the maximum likelihood analyses: a) COI; b) ITS2; c) 28S. Figures represent the relevant subsets of Fig. 3.20, 3.21 and 3.22. Numbers represent significant support values (PP ≥ 95 or BS ≥ 70) for clades recovered in both the Bayesian (PP; top value) and maximum likelihood (BS; bottom value) analyses.

The Sundaicus Complex was also represented by two species, where the species-level resolution for the specimens was relatively poor (Fig. 3.26). Here, specimens were included in Bayesian polytomies,

while *An.* (*Cel.*) *epiroticus* and *An.* (*Cel.*) *sundaicus* were inconsistently recovered as non-monophyletic. The topology was partially associated with specimen geography, since the two Malaysian *An.* (*Cel.*) *sundaicus* specimens clustered together in the COI and ITS2 results.

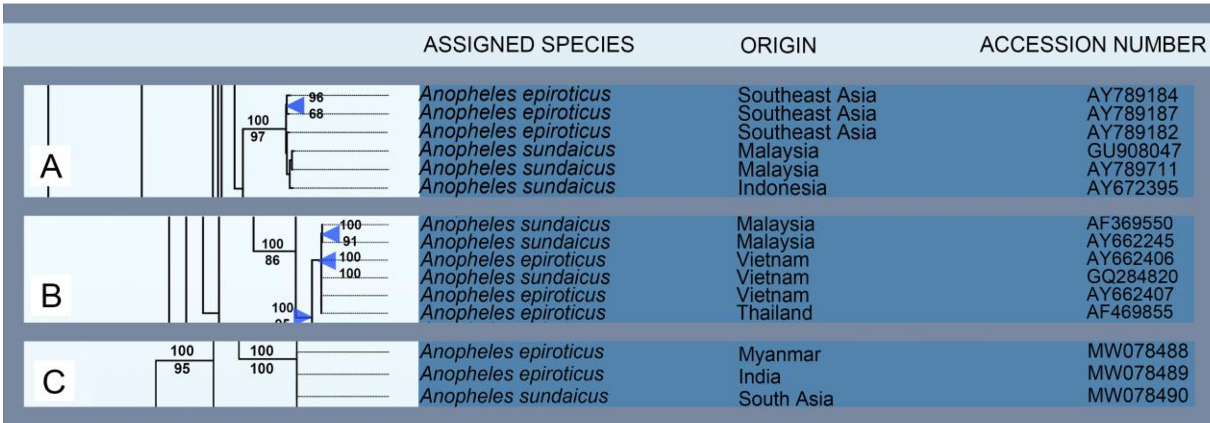

Fig. 3.26. The relationships of the Sundaicus Complex based on the maximum likelihood analyses: a) COI; b) ITS2; c) 28S. Figures represent the relevant subsets of Fig. 3.20, 3.21 and 3.22. Numbers represent significant support values (PP ≥ 95 or BS ≥ 70) for clades recovered in both the Bayesian (PP; top value) and maximum likelihood (BS; bottom value) analyses

Paramyzomyia was also associated with the Pyretophorus Series, yet was only included in the ITS2 dataset. The Paramyzomyia Series was situated medially within the overarching Pyretophorus clade, where both Paramyzomyia and one of its species, *An.* (*Cel.*) *cinereus*, were non-monophyletic (Fig. 3.27). Although this species occurs in South Africa, these specimens were represented by Middle Eastern and Ethiopian specimens, which were situated basally within the Paramyzomyia Series.

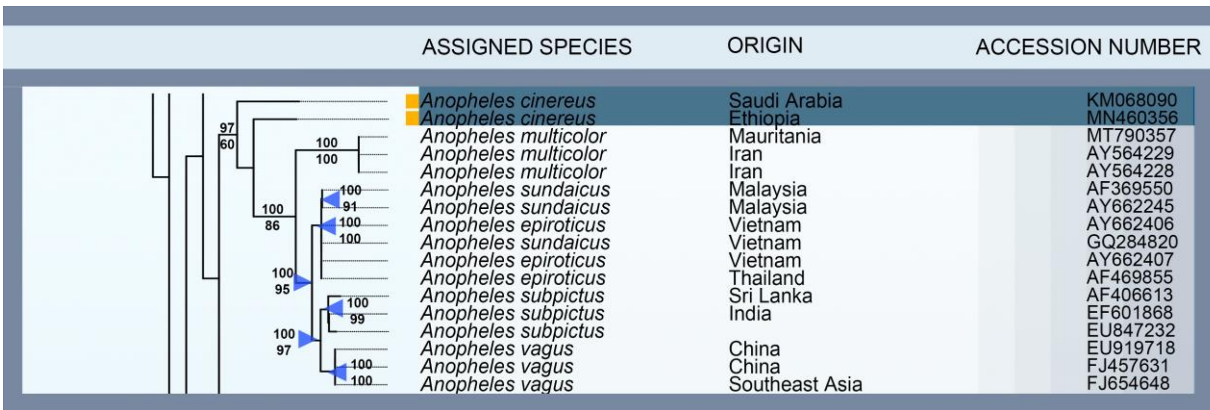

Fig. 3.27. The relationships of the *An.* (*Cel.*) cinereus based on the ITS2 maximum likelihood analysis. The figure represents the relevant subset of Fig. 3.21. Numbers represent significant support values (PP ≥ 95 or BS ≥ 70) for clades recovered in both the Bayesian (PP; top value) and maximum likelihood (BS; bottom value) analyses.

The Myzomyia Series was only polyphyletic in the COI analyses, yet it included two species groups that were non-monophyletic in multiple analyses. Both the Funestus and Demeilloni Groups were polyphyletic and associated with one another in the COI and ITS2 results (Fig. 3.20 & 3.21). These two groups included six South African species, namely *An.* (*Cel.*) *rivulorum*, *An.* (*Cel.*) *leesoni*, *An.* (*Cel.*) *longipalpis*, *An.* (*Cel.*) *parensis*, *An.* (*Cel.*) *funestus* and *An.* (*Cel.*) *demeilloni*. Two Funestus Group

clades remained constant across all applicable analyses, including the monophyletic Funestus Subgroup (COI: PP 100, BS 96; ITS2: PP 100, BS 100) and the clade consisting of the Minimus + Aconitus + Culicifacies Subgroups. Additionally, two species (*An. sergentii* & *An. dthali*) and the Rivulorum Subgroup were consistently situated within the vicinity of the Funestus Subgroup, although not as a monophyletic grouping. Here, *An.* (*Cel.*) *dthali* was the only species that did not belong to either the Demeilloni or Funestus Groups, and was regularly situated within the overarching Funestus Group clade. However, unlike the COI and ITS2 results, the Funestus Group was recovered as a monophyletic clade in the 28S analyses (Fig. 3.22), likely due to the low taxonomic coverage.

The monophyly of the Minimus Subgroup was inconsistent between the DNA regions, due to the placement of one of its species, *An.* (*Cel.*) *flavirostris*. However, the remaining species consistently formed a well-supported grouping consisting of the Minimus Complex + Fluviatilis Complex + *An.* (*Cel.*) *leesoni* (COI: PP 100, BS 98; ITS2: PP 100, BS 93). This relationship between the Minimus and Fluviatilis Complexes was also recovered in the 28S results (PP 89, BS 73).

Two of the *An.* (*Cel.*) *longipalpis* sequences could not be assigned to a specific subgroup, since the publicly available sequences were not designated as either *An.* (*Cel.*) *longipalpis A* or *C*, belonging to the Minimus and Funestus Subgroups, respectively. Both specimens nevertheless clustered with an *An.* (*Cel.*) *longipalpis C* specimen within the Funestus Subgroup, and were therefore considered to be conspecific specimens. The resolution between the various Funestus Subgroup species was generally poor due to the non-monophyly of *An.* (*Cel.*) *longipalpis* in the COI and ITS2 results and the polyphyly of *An.* (*Cel.*) *parensis* in the ITS2 results (Fig. 3.28 a, b). However, *An.* (*Cel.*) *funestus* was the only representative of the subgroup in the 28S analyses.

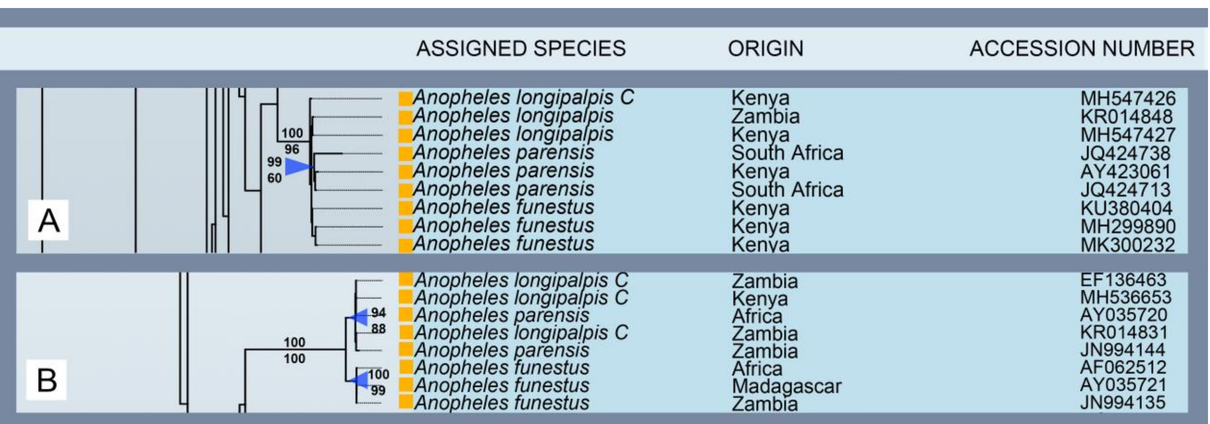

Fig. 3.28. The relationships of the Funestus Subgroup based on the maximum likelihood analyses: a) COI; b) ITS2. Figures represent the relevant subsets of Fig. 3.20 and 3.21. Numbers represent significant support values (PP ≥ 95 or BS ≥ 70) for clades recovered in both the Bayesian (PP; top value) and maximum likelihood (BS; bottom value) analyses.

The affiliations of the two Demeilloni Group species (*An. sergentii* & *An. demeilloni*) were inconsistent between analyses, but were always situated within the overarching Funestus Group clade (Fig. 3.20 & 3.21). The same was true for the Wellcomei and Marshallii Groups, which were included in the ITS2

datasets, where these groups were similarly situated within the Funestus cluster (Fig. 3.21). Here, both representative species (*An. marshallii* & *An. theileri*) occur within South Africa and were not involved in any consistent or well-supported affiliations.

The two remaining Cellia series, Neocellia and Neomyzomyia, were also not included in any consistent species groupings, thus limiting any inferences regarding their internal topology. A few South African species belonged to the Neocellia Series, including *An.* (*Cel.*) *rufipes*, *An.* (*Cel.*) *maculipalpis* and *An.* (*Cel.*) *pretoriensis*. Here, Neocellia's topology was inconsistent between analyses, and it was either recovered as polyphyletic (Fig. 3.20; COI), monophyletic (Fig. 3.21; ITS2: PP 100, BS 100) or was represented by a single specimen (Fig. 3.22; 28S). The monophyly of Neomyzomyia was likewise inconsistent between the COI and ITS2 analyses. A single South African species, *An.* (*Cel.*) *nili* was included in the ITS2 analyses, where its association with the remainder of the Neomyzomyia series was either unsupported or absent.

Subgenus *Nyssorhynchus*

Nyssorhynchus was represented by nine non-South African species, since the subgenus does not occur within the Afrotropical Region. The COI and ITS2 analyses included a relatively large number of species, where the subgenus was consistently recovered as non-monophyletic (Fig. 3.20 & 3.21). However, the subgenus was monophyletic in the 28S results (Fig. 3.22), likely because *Nyssorhynchus* was only represented by two species in this dataset. Most species belonged to one of two sections (Argyritarsis and Albimanus), which were both polyphyletic in the COI and ITS2 results and placed as sister taxa in the smaller 28S analyses (PP 100, BS 97). The polyphyletic sections were largely intermixed with one another, where the intersectional groupings were unsupported and inconsistent. The ITS2 analyses additionally included the Myzorhynchella Section, where it was the most basally situated *Nyssorhynchus* clade.

The sections were further subdivided into several polyphyletic series, including the Argyritarsis, Albitarsis and Oswaldoi Series. These series consisted of numerous scattered clades in the COI and ITS2 analyses, while the 28S results produced a grouping consisting of the Albimanus + Albitarsis Series. The affiliations between these polyphyletic clades were inconsistent and unsupported, and therefore limited any inferences on the relationships of these taxa. Nonetheless, the divisions of *Nyssorhynchus* did not seem to reflect the true evolutionary history of its members, since the majority of sections and series were non-monophyletic.

3.7.3.2 *ANOPHELES* SUBGENERA WITH A LIMITED REPRESENTATION

The large *Anopheles* dataset was divided between relatively few subgenera, and therefore most taxa were represented by numerous species. The only exceptions were two relatively small and underrepresented subgenera (*Kerteszia* & *Stethomyia*). *Kerteszia* was represented by *An.* (*Ker.*)

homunculus in both the COI and ITS2 analyses, where no credible placement was recovered for the subgenus. The affiliations of *Kerteszia* were inconsistent and unsupported, including its weak affiliation with a *Cellia* clade in the COI ML results (Fig. 3.20) and its incorporation into an unsupported *Nyssorhynchus* clade in the ITS2 results (Fig. 3.21). The second subgenus, *Stethomyia* was represented by *An.* (*Ste.*) *kompi* in the COI dataset. Here, both analyses recovered an unsupported clade consisting of *Stethomyia* + *An.* (*Ano.*) *pseudopunctipennis* (Fig. 3.20). However, since few public sequences of *Stethomyia* were available, it was not possible to confirm this association.

3.8 SOUTH AFRICAN HAPLOTYPES

3.8.1 *AEDES*

Aedes (*Stg.*) *aegypti*

Aedes (*Stg.*) *aegypti* was represented by five sequences in the COI dataset, where two consisted of sampled specimens with corroborated identities (AED14.1.1, AED18.11) and three were publicly available sequences (Table 3.5). The dataset included a single database sequence originating from a laboratory colony specimen (Liverpool strain). There was a substantial degree of sequence variation between the two sampled specimens, where numerous single nucleotide polymorphisms (SNPs) were shared between the Bothaville (AED14.1.1) / Chilean specimens and the Bloemfontein (AED18.11) / laboratory colony specimens. Despite the varying affinities, both sampled specimens were morphologically identical. However, no sampled individuals were included in the ITS2 dataset.

Table 3.5. Polymorphic and indel sites for all *Ae.* (*Stg.*) *aegypti* sequences within the COI multiple sequence alignment (11 polymorphic sites / 266 variable sites in the MSA).

Specimen	Locality of origin	30	153	165	231	234	243	348	456	465	477	498
HQ991722	Chile	C	C	A	C	T	A	C	A	A	A	T
HQ991718	Chile	C	C	A	C	T	A	C	A	A	A	T
AED14.1.1	Bothaville	C	T	A	C	T	A	C	A	A	A	C
AED18.11	Bloemfontein Urban Habitat 1	T	C	A	T	C	G	T	A	A	A	C
AY056596	Laboratory Colony	T	C	G	T	C	G	C	G	G	G	C

Aedes (*Och.*) *caballus*

In the COI dataset, *Ae.* (*Och.*) *caballus* was represented by six sampled specimens with corroborated identities (AED18.4, AED18.14, AED19.3, AED19.11, AED20.2707, AED21.1) and three sequences from Iran (Table 3.6). Many alleles were fixed within the South African population, which differentiated these sequences from the Iranian specimens. The sampled specimens all originated from Bloemfontein and several individuals displayed unique polymorphisms, irrespective of their sampling localities within the city. This included one morphologically divergent male with LMS (AED18.4; Fig. 3.6 e), which exhibited a similar number of unique SNPs as its conspecifics.

Within the ITS2 dataset, South African specimens differed substantially from Iranian sequences, which included a gap at positions 571-575 and multiple alleles specific to each country's individuals (Table 3.7). As with the COI data, the South African ITS2 haplotypes were not specific to their sampling sites or associated with specimen morphology (AED18.4, AED19.3, AED19.11, AED20.2707, AED21.1). Here, the atypical male (AED18.4) exhibited no unique polymorphisms, while a morphologically typical specimen (AED20.2707) had a unique insertion at positions 537-538. Therefore, both datasets consisted of genetically distinct specimens based on their country of origin, while there was no evident pattern to the occurrence of South African alleles.

Table 3.6. Polymorphic and indel sites for all *Ae.* (*Och.*) *caballus* sequences within the COI multiple sequence alignment (29 polymorphic sites / 266 variable sites in the MSA). Missing data are indicated with a question mark.

Specimen	Locality of origin	42	156	205	231	234	258	264	267	270	273	279	315	318	321	354
MH634431	Iran	T	T	C	C	T	T	A	A	T	T	G	A	A	G	T
MH634434	Iran	T	T	C	C	T	T	A	A	T	T	G	A	A	G	T
MH709107	Iran	T	T	C	C	T	T	A	A	T	T	G	A	A	G	T
AED18.4	Bloemfontein Urban Habitat 1	A	T	T	T	C	C	G	A	C	A	A	A	A	G	T
AED19.11	Bloemfontein Urban Habitat 2	A	T	T	T	C	C	G	G	C	A	A	A	A	G	T
AED19.3	Bloemfontein Urban Habitat 2	A	T	T	T	C	C	G	G	C	A	A	G	A	G	T
AED21.1	Bloemfontein Urban Habitat 3	A	T	T	T	C	C	G	A	T	A	A	G	A	G	T
AED20.2707	Bloemfontein Semi-Pristine Habitat 2	A	T	T	T	C	C	G	A	C	A	A	A	A	G	G
AED18.14	Bloemfontein Urban Habitat 1	A	C	T	T	C	C	G	A	C	A	A	A	G	A	T

Specimen	Locality of origin	357	363	369	381	384	411	472	474	477	489	561	577	585	588
MH634431	Iran	A	C	A	A	C	G	G	A	A	C	C	T	T	T
MH634434	Iran	A	C	A	A	C	G	G	A	A	C	C	T	T	T
MH709107	Iran	A	C	A	A	C	A	G	A	A	C	C	T	T	T
AED18.4	Bloemfontein Urban Habitat 1	A	G	G	T	T	T	T	C	T	T	C	?	?	?
AED19.11	Bloemfontein Urban Habitat 2	A	A	A	T	T	T	T	T	T	T	C	C	?	?
AED19.3	Bloemfontein Urban Habitat 2	A	A	A	T	T	T	T	T	T	T	?	?	?	?
AED21.1	Bloemfontein Urban Habitat 3	G	A	A	T	T	T	T	T	T	T	?	?	?	?
AED20.2707	Bloemfontein Semi-Pristine Habitat 2	A	A	G	T	T	T	T	T	T	T	?	?	?	?
AED18.14	Bloemfontein Urban Habitat 1	A	A	G	T	T	T	T	T	T	T	G	C	A	C

Table 3.7. Polymorphic and indel sites for all *Ae.* (*Och.*) *caballus* sequences within the ITS2 multiple sequence alignment (30 polymorphic and indel sites / 328 variable sites in the MSA). Ambiguous nucleotides are indicated with an 'N' and gaps with a dash.

Specimen	Locality of origin	52	77	266	268	270	279	286	490	496	513	527	528	537	538	542
MN158185	Iran	C	T	C	G	C	G	C	G	T	G	A	T	-	-	C
MN158188	Iran	C	T	C	G	C	G	C	G	T	G	A	T	-	-	C
MN158189	Iran	C	T	C	G	C	G	C	G	T	G	A	T	-	-	C
AED18.4	Bloemfontein Urban Habitat 1	T	C	T	C	T	T	T	G	T	N	C	N	-	-	A
AED19.3	Bloemfontein Urban Habitat 2	T	C	T	C	T	T	T	G	T	G	C	A	-	-	A
AED19.11	Bloemfontein Urban Habitat 2	T	C	T	C	T	T	T	G	T	G	C	T	-	-	A
AED21.1	Bloemfontein Urban Habitat 3	T	C	T	C	T	T	T	N	G	T	C	T	-	-	A
AED20.2707	Bloemfontein Semi-Pristine Habitat 2	T	C	T	C	T	T	T	C	G	T	C	T	C	A	A

Specimen	Locality of origin	544	571	572	573	574	575	576	577	578	580	583	586	587	588	589
MN158185	Iran	A	T	C	G	A	C	A	T	T	C	T	-	-	A	C
MN158188	Iran	A	T	C	G	A	C	A	T	T	C	T	A	C	A	C
MN158189	Iran	A	T	C	G	A	C	A	T	T	C	T	-	-	A	C
AED18.4	Bloemfontein Urban Habitat 1	C	-	-	-	-	-	G	G	A	G	A	-	-	T	C
AED19.3	Bloemfontein Urban Habitat 2	C	-	-	-	-	-	G	G	A	G	A	-	-	T	C
AED19.11	Bloemfontein Urban Habitat 2	N	-	-	-	-	-	T	N	A	N	A	-	-	T	A
AED21.1	Bloemfontein Urban Habitat 3	N	-	-	-	-	-	T	N	A	N	A	-	-	T	A
AED20.2707	Bloemfontein Semi-Pristine Habitat 2	C	-	-	-	-	-	G	G	A	G	A	-	-	T	C

Aedes* (*Och.*) *juppi

Aedes (*Och.*) *juppi* was represented by six sequenced specimens with corroborated identities (AED2.1, AED9.1, AED12.1, AED18.9, AED20.107, AED20.2704) in the COI dataset (Table 3.8). The haplotypes were not noticeably associated with their biogeography, and numerous SNPs (positions 30, 258, 552) were unique to a single Bloemfontein specimen (AED20.2704). Despite these sequence variations, no morphological variation was noted in any sequenced specimens. Two Bloemfontein specimens were also included in the ITS2 dataset (AED18.9, AED20.2704), where no nucleotide polymorphisms were observed (results not shown).

Table 3.8. Polymorphic and indel sites for all *Ae.* (*Och.*) *juppi* sequences within the COI multiple sequence alignment (7 polymorphic sites / 266 variable sites in the MSA). Ambiguous nucleotides are indicated with an 'N' and missing data with a question mark.

Specimen	Locality of origin	30	81	87	258	414	552	570
AED12.1	Memel	G	A	G	C	A	T	T
AED18.9	Bloemfontein Urban Habitat 1	G	N	A	C	A	T	T
AED9.1	Bethulie	G	A	A	C	G	T	T
AED2.1	Bloemfontein Smallholding 2	G	A	A	C	G	T	G
AED20.107	Bloemfontein Semi-Pristine Habitat 2	G	G	A	N	G	?	?
AED20.2704	Bloemfontein Semi-Pristine Habitat 2	A	N	A	T	G	G	T

Aedes (*Neo.*) *mcintoshi*

Ae (*Neo.*) *mcintoshi* was represented by three sampled specimens with corroborated identities (AED20.2710, AED20.405, AED22.19) and three non-sampled specimens in the COI dataset (Table 3.9). Here, no alleles differentiated the South African specimens from other African sequences. Each South African specimen displayed a unique array of SNPs, and haplotypes were not shared between specimens from the same locality. The *Ae.* (*Neo.*) cf. *mcintoshi* specimen with the atypically light coloured sterna (AED20.405; Fig. 3.6 c) also did not possess an unusual amount of unique polymorphisms compared to its conspecifics.

In the ITS2 dataset, the species was represented by two sampled Bloemfontein specimens (AED20.2710, AED20.405) and three Kenyan specimens (Table 3.10). Here, many alleles and several gaps differentiated the sampled specimens from other African sequences. Also, despite the unusual morphology of specimen AED20.405, no ITS2 sequence variation was observed between the two sampled specimens. Therefore, the two DNA regions yielded contradicting profiles, since the ITS2 region consisted of genetically distinct populations of specimens based on their country of origin, which was not observed in the COI region. Furthermore, there was no apparent pattern to the occurrence of South African alleles in any of the datasets.

Table 3.9. Polymorphic and indel sites for all *Ae.* (*Neo.*) *mcintoshi* sequences within the COI multiple sequence alignment (49 polymorphic sites / 266 variable sites in the MSA). Ambiguous nucleotides are indicated with an 'N' and missing data with a question mark.

Specimen	Locality of origin																	
		1	39	84	93	117	144	156	168	171	180	186	198	216	228	234	258	265
KJ940679	Kenya	?	G	A	C	T	C	T	T	T	A	C	A	A	G	C	C	T
KJ940551	Kenya	?	A	T	T	C	T	C	A	T	A	T	A	A	A	T	T	C
LC473696	Malawi	A	G	T	T	C	T	C	A	T	A	T	G	A	A	T	T	C
AED20.405	Bloemfontein Semi-Pristine Habitat 2	A	G	T	T	C	T	C	A	T	A	T	A	A	A	T	T	C
AED20.2710	Bloemfontein Semi-Pristine Habitat 2	A	A	T	T	N	T	C	A	T	A	T	A	G	A	T	T	C
AED22.19	Bloemfontein Semi-Pristine Habitat 3	G	A	T	T	C	T	N	A	G	G	T	A	A	A	T	T	C
		267	270	273	279	285	315	321	327	346	357	369	372	373	396	411	432	
KJ940679	Kenya	A	T	T	G	A	A	A	T	C	A	T	G	G	C	A	A	
KJ940551	Kenya	A	T	C	A	T	G	A	A	T	T	A	A	G	T	A	A	
LC473696	Malawi	T	T	T	A	A	A	A	G	C	T	T	A	A	T	A	A	
AED20.405	Bloemfontein Semi-Pristine Habitat 2	T	C	C	A	A	A	G	G	C	T	T	A	G	T	A	G	
AED20.2710	Bloemfontein Semi-Pristine Habitat 2	T	T	C	G	A	A	A	A	C	T	T	G	G	T	A	A	
AED22.19	Bloemfontein Semi-Pristine Habitat 3	T	T	C	N	A	G	A	A	C	T	T	G	G	T	G	A	
		435	459	465	471	474	495	507	561	564	567	576	606	612	615	624	633	
KJ940679	Kenya	C	T	A	C	A	A	A	A	A	C	G	T	T	T	A	C	
KJ940551	Kenya	T	T	A	T	A	A	T	C	A	T	A	A	C	C	G	T	
LC473696	Malawi	C	T	A	T	G	A	T	C	A	T	A	A	C	C	A	C	
AED20.405	Bloemfontein Semi-Pristine Habitat 2	C	T	A	T	A	A	T	T	G	T	A	A	C	C	A	C	
AED20.2710	Bloemfontein Semi-Pristine Habitat 2	C	C	G	T	A	G	T	C	A	T	?	?	?	?	?	?	
AED22.19	Bloemfontein Semi-Pristine Habitat 3	C	T	N	T	A	A	T	C	?	?	?	?	?	?	?	?	

Table 3.10. Polymorphic and indel sites for all *Ae.* (*Neo.*) *mcintoshi* sequences within the ITS2 multiple sequence alignment (42 polymorphic and indel sites / 328 variable sites in the MSA). Ambiguous nucleotides are indicated with an 'N' and gaps with a dash.

Specimen	Locality of origin	53	157	158	159	160	161	163	262	263	272	273	274	275	276
KJ940769	Kenya	G	-	-	-	-	C	T	T	G	C	T	G	G	T
KJ940770	Kenya	G	-	-	-	-	C	T	T	G	C	C	G	G	T
KJ940771	Kenya	G	-	-	-	-	C	T	T	G	C	C	G	G	T
AED20.405	Bloemfontein Semi-Pristine Habitat 2	C	T	C	C	G	G	G	C	-	-	-	-	-	-
AED20.2710	Bloemfontein Semi-Pristine Habitat 2	C	T	C	C	G	G	G	C	-	-	-	-	-	-

Specimen	Locality of origin	277	278	279	280	284	538	539	540	541	542	543	544	568	569
KJ940769	Kenya	A	C	G	G	T	-	-	-	-	-	-	-	-	-
KJ940770	Kenya	A	C	G	G	T	-	-	-	-	-	-	-	-	-
KJ940771	Kenya	A	C	G	G	T	-	-	-	-	-	-	-	-	-
AED20.405	Bloemfontein Semi-Pristine Habitat 2	-	-	-	-	G	A	N	N	A	C	C	C	N	T
AED20.2710	Bloemfontein Semi-Pristine Habitat 2	-	-	-	-	G	A	N	C	A	C	C	C	C	T

Specimen	Locality of origin	570	571	572	574	576	577	579	580	581	582	588	589	590	591
KJ940769	Kenya	-	-	A	C	C	T	G	T	G	G	A	C	T	G
KJ940770	Kenya	-	-	A	C	C	T	G	T	G	G	A	C	T	G
KJ940771	Kenya	-	-	A	C	C	T	G	T	G	G	A	C	T	G
AED20.405	Bloemfontein Semi-Pristine Habitat 2	G	T	G	A	T	G	T	A	T	A	C	T	C	A
AED20.2710	Bloemfontein Semi-Pristine Habitat 2	G	T	G	A	T	G	T	A	T	A	C	T	C	A

Aedes (*Neo.*) *unidentatus*

Aedes (*Neo.*) *unidentatus* was represented by two sampled specimens with corroborated identities in the COI dataset (AED8.30, AED22.18), which were collected at Bethlehem and Bloemfontein (Table 3.11). The sequences differed at seven loci, although both specimens were morphologically similar. Since only two specimens were included in the dataset, it was not possible to determine whether the polymorphisms were a product of population-level or individual variation.

Table 3.11. Polymorphic and indel sites for all *Ae.* (*Neo.*) *unidentatus* sequences within the COI multiple sequence alignment (7 polymorphic sites / 266 variable sites in the MSA).

Specimen	Locality of origin	39	198	234	327	387	432	499
AED8.30	Bethlehem	G	G	T	G	A	A	T
AED22.18	Bloemfontein Semi-Pristine Habitat 3	A	A	C	A	G	G	C

3.8.2 *CULEX*

Culex (*Cux.*) *pipiens*

Culex (*Cux.*) *pipiens* was represented by ten sequences in the COI dataset, consisting of seven sampled individuals with corroborated identities (CLX22.42, CLX2.82, CLX2.280, CLX21.10, CLX2.90, CLX5.2.7, CLX12.10) and three Eurasian sequences (Table 3.12). South African and Eurasian specimens were distinguished by numerous fixed alleles, while several morphologically typical and

atypical sampled individuals sporadically possessed unique nucleotide polymorphisms, including one relatively dark and robust specimen (CLX12.10) from Memel. Numerous haplotypes were also observed across the multiple Bloemfontein sampling sites.

However, the ITS2 region displayed minimal sequence variation, where polymorphic and indel sites were restricted to four positions in the MSA. The dataset consisted of two sampled individuals (CLX2.280, CLX21.10), one non-sampled South African sequence and one specimen from a laboratory colony (Table 3.13). The two sampled specimens were collected in Bloemfontein, and despite their morphological similarity, each possessed a unique haplotype. Although no alleles were fixed in all South African specimens, specimens still possessed gaps at positions 175 and 313 that were not present in the laboratory colony specimen. Therefore, South African specimens were inherently distinct in both datasets, while there was no apparent pattern to the occurrence of their alleles.

Table 3.12. Polymorphic and indel sites for all *Cx.* (*Cux.*) *pipiens* sequences within the COI multiple sequence alignment (22 polymorphic sites / 250 variable sites in the MSA). Ambiguous nucleotides are indicated with an 'N' and missing data with a question mark.

Specimen	Locality of origin	22	31	38	61	85	172	211	232	259	289	316
JQ253847	United Kingdom	?	T	G	A	A	C	G	C	T	A	A
AM403476	Russia	?	T	G	A	A	C	G	C	T	A	A
FN395181	Russia	G	T	G	A	A	C	G	C	T	A	A
CLX22.42	Bloemfontein Semi-Pristine Habitat 3	A	C	A	T	C	T	A	T	T	C	G
CLX2.82	Bloemfontein Smallholding 2	?	?	?	T	C	T	A	T	T	C	A
CLX2.280	Bloemfontein Smallholding 2	A	T	A	T	C	T	A	T	T	A	A
CLX21.10	Bloemfontein Urban Habitat 3	A	T	A	T	C	T	A	T	T	A	A
CLX2.90	Bloemfontein Smallholding 2	A	T	A	T	C	T	A	T	T	A	A
CLX5.2.7	Gariep Dam Nature Reserve	A	N	A	T	C	T	A	T	T	A	A
CLX12.10	Memel	A	N	A	T	C	T	A	T	C	C	A

Specimen	Locality of origin	322	385	457	466	472	499	562	565	616	628	634
JQ253847	United Kingdom	G	C	A	A	T	T	T	T	T	?	?
AM403476	Russia	G	C	A	A	T	T	T	T	T	T	?
FN395181	Russia	G	C	A	A	T	T	T	T	T	A	T
CLX22.42	Bloemfontein Semi-Pristine Habitat 3	A	T	A	A	A	A	C	A	?	?	?
CLX2.82	Bloemfontein Smallholding 2	A	T	G	G	A	A	?	?	?	?	?
CLX2.280	Bloemfontein Smallholding 2	A	T	A	G	A	A	?	?	?	?	?
CLX21.10	Bloemfontein Urban Habitat 3	A	T	G	G	A	A	C	A	?	?	?
CLX2.90	Bloemfontein Smallholding 2	A	T	G	G	A	A	C	A	?	?	?
CLX5.2.7	Gariep Dam Nature Reserve	A	T	G	G	A	A	C	A	C	A	C
CLX12.10	Memel	A	T	G	G	A	A	C	A	?	?	?

Table 3.13. Polymorphic and indel sites for all *Cx.* (*Cux.*) *pipiens* sequences within the ITS2 multiple sequence alignment (4 polymorphic and indel sites / 333 variable sites in the MSA). Gaps are indicated with a dash.

Specimen	Locality of origin	175	244	282	313
CLX21.10	Bloemfontein Urban Habitat 3	-	A	G	-
CLX2.280	Bloemfontein Smallholding 2	-	G	C	-
DQ341109	South Africa	-	G	C	-
U22115	Laboratory Colony	G	G	C	A

Culex (*Cux.*) *quinquefasciatus*

Culex (*Cux.*) *quinquefasciatus* was represented by five sampled specimens with corroborated identities (CLX4.2.19, CLX13.5, CLX19.2, CLX18.2, CLX21.5) and three publicly available sequences in the COI dataset (Table 3.14). Three positions within the MSA were polymorphic, where alleles were not associated with the specimen's country of origin. It was also not apparent whether haplotypes were a product of the specimens' locality in the Free State Province, since the three Bloemfontein specimens did not share a haplotype. However, one haplotype was shared between Ugandan, Indian and two of the Bloemfontein specimens. On the other hand, the Willem Pretorius Game Reserve and Oranjeville specimens each displayed unique SNPs. Here, the Willem Pretorius Game Reserve specimen (CLX4.2.19) also exhibited underdeveloped tergal bands (Fig. 3.6 i), while the Oranjeville specimen (CLX13.5) was relatively small and delicate, which might have contributed to the presence of their unique polymorphisms.

Table 3.14. Polymorphic and indel sites for all *Cx.* (*Cux.*) *quinquefasciatus* specimens within the COI multiple sequence alignment (3 polymorphic sites / 250 variable sites in the MSA).

Specimen	Locality of origin			
		283	316	448
GQ165796	Uganda	A	G	T
FN395201	India	A	G	T
FM177756	India	A	G	T
CLX4.2.19	Willem Pretorius Game Reserve	A	A	T
CLX19.2	Bloemfontein Urban Habitat 2	A	G	T
CLX18.2	Bloemfontein Urban Habitat 1	A	G	T
CLX21.5	Bloemfontein Urban Habitat 3	A	G	C
CLX13.5	Oranjeville	T	G	C

Culex (*Cux.*) *theileri*

In the COI dataset, *Cx.* (*Cux.*) *theileri* was represented by five sampled specimens with corroborated identities (CLX2.24, CLX1.11, CLX1.6, CLX15.1, CLX4.2.5) and three Eurasian sequences (Table 3.15). The South African specimens were characterised by numerous stable alleles, while only a single specimen from Bloemfontein smallholding 1 exhibited a unique SNP (CLX1.6). This haplotype was not population-specific, since another specimen from the same locality displayed the typical haplotype shared by all remaining specimens. Since most specimens shared a haplotype, morphological variation was generally not associated with any alleles, except for the relatively light coloured specimen (CLX1.6) that displayed a single SNP.

The ITS2 dataset was once again represented by sampled specimens with corroborated identities (CLX4.2.5, CLX2.21, CLX2.24) and three Eurasian sequences (Table 3.16). South African specimens possessed multiple distinct alleles in addition to gaps between positions 231-241. There was once again no apparent association between the haplotypes and their sampling locality. Here, two specimens from the same site displayed numerous sequence differences (CLX2.21, CLX2.24) and also possessed

morphological differences in their abdominal patterning (Fig. 3.6 k, l). Therefore, the South African sequences from both datasets were distinct, while the region's alleles may have either reflected individual variation or morphological differences.

Table 3.15. Polymorphic and indel sites for all *Cx.* (*Cux.*) *theileri* sequences within the COI multiple sequence alignment (9 polymorphic sites / 250 variable sites in the MSA). Missing data are indicated with a question mark.

Specimen	Locality of origin	10	208	220	283	322	361	433	562	578
FJ210898	Iran	T	A	A	A	A	A	A	T	T
HE610457	Portugal	T	A	A	A	A	A	A	T	T
JN051388	Spain	T	A	A	A	G	A	T	T	T
CLX2.24	Bloemfontein Smallholding 2	C	G	T	T	A	A	A	?	?
CLX1.11	Bloemfontein Smallholding 1	?	G	T	T	A	A	A	C	C
CLX1.6	Bloemfontein Smallholding 1	?	G	T	T	A	C	A	C	?
CLX15.1	Boshof	C	G	T	T	A	A	A	C	C
CLX4.2.5	Willem Pretorius Game Reserve	C	G	T	T	A	A	A	C	C

Table 3.16. Polymorphic and indel sites for all *Cx.* (*Cux.*) *theileri* sequences within the ITS2 multiple sequence alignment (65 polymorphic and indel sites / 333 variable sites in the MSA). Ambiguous nucleotides are indicated with an 'N', missing data with a question mark and gaps with a dash.

Specimen	Locality of origin																	
		24	67	68	69	70	105	114	151	168	193	194	197	231	237	238	239	240
MF288849	China	A	-	-	-	-	A	G	C	C	A	C	C	G	A	G	A	C
JN051384	Spain	G	G	C	G	C	G	G	C	T	C	A	C	G	A	C	A	C
KF483841	Iran	G	G	C	G	C	G	A	G	T	C	A	T	G	A	C	A	C
CLX4.2.5	Willem Pretorius Game Reserve	G	G	C	G	C	G	G	C	T	C	A	C	-	-	-	-	-
CLX2.21	Bloemfontein Smallholding 2	G	G	C	G	C	G	G	C	T	C	A	C	-	-	-	-	-
CLX2.24	Bloemfontein Smallholding 2	G	G	C	G	C	G	G	C	T	C	A	C	-	-	-	-	-
		241	276	282	288	309	312	313	314	315	316	322	323	332	333	334	335	
MF288849	China	A	A	C	C	T	G	C	-	-	-	-	-	G	T	C	G	
JN051384	Spain	-	A	C	C	T	C	T	C	T	C	T	G	-	-	-	-	
KF483841	Iran	-	G	G	A	T	C	T	-	-	-	-	G	-	-	-	-	
CLX4.2.5	Willem Pretorius Game Reserve	-	A	C	C	A	T	T	-	-	-	-	G	G	N	T	N	
CLX2.21	Bloemfontein Smallholding 2	-	A	C	C	A	T	T	-	-	-	-	G	G	C	T	G	
CLX2.24	Bloemfontein Smallholding 2	-	A	C	C	A	T	T	-	-	-	-	G	G	C	T	G	
		336	337	338	339	396	402	403	404	407	408	414	416	417	418	419	420	
MF288849	China	G	T	G	G	C	G	A	A	C	C	G	G	C	-	-	-	
JN051384	Spain	-	-	-	-	C	A	A	G	-	-	G	G	A	A	C	C	
KF483841	Iran	-	-	-	-	A	-	-	-	-	-	A	A	C	C	C	C	
CLX4.2.5	Willem Pretorius Game Reserve	C	T	-	G	?	?	?	?	?	?	?	?	?	?	?	?	
CLX2.21	Bloemfontein Smallholding 2	C	T	-	G	C	G	A	-	-	-	N	A	N	A	C	C	
CLX2.24	Bloemfontein Smallholding 2	C	T	-	G	C	G	A	-	-	-	G	N	A	A	C	C	
		421	422	423	424	425	426	427	431	433	434	435	446	447	448	449	452	
MF288849	China	-	-	-	-	-	-	A	T	G	A	T	-	A	C	A	A	
JN051384	Spain	C	C	C	?	?	?	?	?	?	?	?	?	?	?	?	?	
KF483841	Iran	C	A	C	-	-	-	A	T	G	A	-	-	-	T	A	C	
CLX4.2.5	Willem Pretorius Game Reserve	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	
CLX2.21	Bloemfontein Smallholding 2	C	C	C	N	A	N	N	A	A	T	N	A	C	C	C	C	
CLX2.24	Bloemfontein Smallholding 2	C	C	C	A	C	N	C	T	G	A	T	A	C	C	A	C	

Culex (*Cux.*) *univittatus*

Culex (*Cux.*) *univittatus* was represented by many sequences in the COI dataset, including 13 sampled specimens with corroborated identities, two non-sampled South African specimens and one Malawian sequence (Table 3.17). All South African specimens shared multiple alleles, which differed from the single Malawian specimen. Additionally, a group of four sampled specimens (CLX18.3, CLX1.16, CLX9.4, CLX4.2.9) and a South African specimen from the Kruger National Park (KMPED801-18) shared alleles at three positions (1, 100, 412). The remaining sampled specimens (CLX12.3, CLX12.5, CLX2.172, CLX2.256, CLX2.68, CLX2.69, CLX4.2.1, CLX7.4, CLX8.3) shared their alleles with a South African specimen from the Gauteng Province (GMSAB076-13). These South African polymorphisms occurred independent of their associated geography, and some specimens from the same region differed substantially. Furthermore, a large degree of morphological variation was observed within *Cx.* (*Cux.*) *univittatus*, and most specimens presented with a unique set of morphological characteristics (Fig. 3.6 m–r). One relatively dark specimen (CLX2.69) possessed a shorter stripe on the hindfemur (Fig. 3.6 q), which coincided with several unique SNPs. Another specimen (CLX2.172) with a relatively large postspiracular scale patch (Fig. 3.6 m) also presented with a single unique SNP.

Table 3.17. Polymorphic and indel sites for all *Cx.* (*Cux.*) *univittatus* sequences within the COI multiple sequence alignment (21 polymorphic sites / 250 variable sites in the MSA). Ambiguous nucleotides are indicated with an 'N' and missing data with a question mark.

Specimen	Locality of origin	1	28	38	100	109	128	142	190	217	220	229
LC473638	Malawi	A	A	A	A	A	A	T	A	G	C	G
GMSAB076-13	South Africa	A	A	A	G	A	A	C	A	A	T	G
KMPED801-18	South Africa	?	A	A	A	A	A	C	A	A	T	G
CLX18.3	Bloemfontein Urban Habitat 1	G	A	A	A	A	N	C	A	A	T	G
CLX1.16	Bloemfontein Smallholding 1	G	A	A	A	A	A	C	A	A	T	G
CLX9.4	Bethulie	G	A	A	A	A	A	C	A	A	T	G
CLX4.2.9	Willem Pretorius Game Reserve	G	G	A	A	A	A	C	A	A	T	G
CLX12.3	Memel	A	A	A	G	A	A	C	A	A	T	G
CLX7.4	Rosendal	?	?	?	G	N	A	C	A	A	T	G
CLX8.3	Bethlehem	A	A	A	G	A	A	C	A	A	T	G
CLX12.5	Memel	A	A	A	G	G	A	C	A	A	T	G
CLX2.68	Bloemfontein Smallholding 2	A	A	A	G	A	N	C	A	A	T	G
CLX2.256	Bloemfontein Smallholding 2	A	A	G	G	A	A	C	A	A	T	G
CLX4.2.1	Willem Pretorius Game Reserve	A	A	A	G	A	C	C	A	A	T	G
CLX2.172	Bloemfontein Smallholding 2	A	A	A	G	A	N	C	A	A	T	G
CLX2.69	Bloemfontein Smallholding 2	A	A	A	G	A	A	T	G	A	T	A

Specimen	Locality of origin	271	286	289	412	433	496	526	544	625	634
LC473638	Malawi	T	A	A	T	G	A	C	G	A	C
GMSAB076-13	South Africa	T	A	A	T	A	A	T	A	?	?
KMPED801-18	South Africa	T	A	A	C	A	A	T	A	A	C
CLX18.3	Bloemfontein Urban Habitat 1	T	A	A	C	A	A	T	A	?	?
CLX1.16	Bloemfontein Smallholding 1	T	G	A	C	A	A	T	A	A	C
CLX9.4	Bethulie	T	A	A	C	A	A	T	A	?	?
CLX4.2.9	Willem Pretorius Game Reserve	T	A	A	C	A	A	T	A	A	?
CLX12.3	Memel	T	A	A	T	A	A	T	A	A	T
CLX7.4	Rosendal	T	N	N	T	A	A	?	?	?	?
CLX8.3	Bethlehem	T	A	A	T	A	A	T	A	A	T
CLX12.5	Memel	T	A	A	T	A	A	T	A	A	T
CLX2.68	Bloemfontein Smallholding 2	T	A	A	T	A	A	T	A	A	T
CLX2.256	Bloemfontein Smallholding 2	C	A	A	T	A	A	T	A	A	T
CLX4.2.1	Willem Pretorius Game Reserve	T	N	C	T	A	A	T	A	?	?
CLX2.172	Bloemfontein Smallholding 2	T	C	C	T	A	A	T	A	?	?
CLX2.69	Bloemfontein Smallholding 2	T	A	A	T	A	G	T	A	G	C

3.8.3 *ANOPHELES*

Anopheles (*Cel.*) *squamosus*

Six Squamosus Group specimens were added to the COI dataset, including three non-sampled African *An.* (*Cel.*) *squamosus* sequences, two morphologically similar sampled *An.* (*Cel.*) *cydippis* / *squamosus* specimens (ANO7.1, ANO22.1) and one damaged *Anopheles* sp. specimen (ANO3.1). The damaged specimen's BLAST results were nonetheless comparable to those of the *An.* (*Cel.*) *cydippis* / *squamosus* individuals, and it was therefore treated as a conspecific individual. Several alleles were shared between the sampled South African specimens, which distinguished these sequences from the other wide-ranging African specimens (Table 3.18). Since few alleles were shared between the South African and non-South African populations, the sequences demonstrated a large degree of genetic

separation. Furthermore, despite their shared morphology, the sampled specimens possessed three unique haplotypes, irrespective of their origin within the Free State.

Within the ITS2 dataset, the Squamosus Group was represented by a damaged *Anopheles* specimen (ANO3.1) and three other African *An.* (*Cel.*) *squamosus* sequences (Table 3.19). The South African specimen once again differed substantially from all other *An.* (*Cel.*) *squamosus* specimens, which included several unique nucleotide polymorphisms and insertions at positions 1,594-1,598 and 1,844-1,847. Therefore, both DNA regions yielded genetically distinct South African specimens when compared to non-South African *An.* (*Cel.*) *squamosus* sequences.

Table 3.18. Polymorphic and indel sites for all Squamosus Group sequences within the COI multiple sequence alignment (37 polymorphic sites / 280 variable sites in the MSA). Ambiguous nucleotides are indicated with an 'N' and missing data with a question mark.

Specimen	Locality of origin																			
		4	55	79	82	121	124	127	136	148	154	181	187	212	220	229	235	238	259	271
MK586052	Mali	A	T	T	C	A	T	T	T	G	C	A	T	C	T	G	T	T	C	T
KJ522841	Kenya	?	T	T	C	G	T	T	T	G	C	A	T	C	T	A	T	T	T	T
LC473607	Malawi	A	A	T	C	G	T	T	T	G	C	A	T	C	T	G	T	T	T	T
ANO22.1	Bloemfontein Semi-Pristine Habitat 3	T	T	G	T	A	N	A	N	A	T	G	N	T	C	A	C	T	N	T
ANO3.1	Bloemfontein Semi-Pristine Habitat 1	?	?	N	T	N	N	A	C	A	T	G	N	T	C	A	C	C	T	T
ANO7.1	Rosendal	?	?	A	T	A	C	A	C	A	T	G	C	T	C	A	C	T	T	C
		283	292	316	343	346	364	367	373	397	412	475	485	514	539	556	558	562	568	
MK586052	Mali	T	T	A	T	A	T	T	G	T	T	T	T	T	C	T	T	T	A	
KJ522841	Kenya	T	T	A	T	A	T	T	G	T	A	T	T	T	C	T	T	T	A	
LC473607	Malawi	T	T	A	T	A	C	T	A	T	A	T	T	C	C	T	T	T	A	
ANO22.1	Bloemfontein Semi-Pristine Habitat 3	A	C	G	C	T	T	N	A	C	A	C	N	N	N	?	?	?	?	
ANO3.1	Bloemfontein Semi-Pristine Habitat 1	A	C	G	C	T	T	C	A	C	A	N	N	T	T	A	G	A	T	
ANO7.1	Rosendal	A	C	A	C	T	T	C	A	C	A	N	C	T	T	A	T	A	A	

Table 3.19. Polymorphic and indel sites for all Squamosus Group sequences within the ITS2 multiple sequence alignment (77 polymorphic and indel sites / 1,389 variable sites in the MSA). Ambiguous nucleotides are indicated with an 'N', missing data with a question mark and gaps with a dash.

Specimen	Locality of origin	24	146	293	294	432	518	699	700	778	780	1123	1124	1253	1494	1495	1514
ANO3.1	Bloemfontein Semi-Pristine Habitat 1	A	T	T	T	C	T	C	T	T	G	G	T	G	C	A	G
MK592071	Africa	A	A	T	T	C	T	C	T	T	G	G	T	G	T	G	T
KJ522825	Kenya	C	A	A	C	T	A	T	A	-	A	-	-	A	T	G	T
KR014825	Zambia	A	A	A	C	T	A	T	A	-	A	-	-	A	T	G	T

Specimen	Locality of origin	1516	1521	1522	1523	1524	1525	1526	1527	1581	1582	1583	1587	1588	1589	1590	1591
ANO3.1	Bloemfontein Semi-Pristine Habitat 1	A	G	T	G	A	A	G	T	G	G	C	T	T	T	G	C
MK592071	Africa	G	C	G	C	A	A	G	T	G	T	T	G	C	T	C	A
KJ522825	Kenya	A	-	-	-	-	G	T	G	A	T	T	G	C	T	C	T
KR014825	Zambia	A	-	-	-	-	G	T	G	A	T	T	N	C	T	C	T

Specimen	Locality of origin	1592	1593	1594	1595	1596	1597	1598	1616	1617	1644	1673	1737	1755	1757	1791
ANO3.1	Bloemfontein Semi-Pristine Habitat 1	T	A	C	C	T	A	C	C	C	-	T	T	C	T	N
MK592071	Africa	C	C	-	-	-	-	-	C	G	-	T	T	T	T	G
KJ522825	Kenya	A	C	-	-	-	-	-	C	G	C	G	A	G	C	A
KR014825	Zambia	A	C	-	-	-	-	-	C	G	C	G	?	?	?	?

Specimen	Locality of origin	1792	1793	1794	1795	1796	1833	1844	1845	1846	1847	1873	1875	1879	1883	1884
ANO3.1	Bloemfontein Semi-Pristine Habitat 1	C	C	A	A	G	G	N	G	G	N	G	T	A	A	C
MK592071	Africa	C	C	A	A	G	A	-	-	-	-	A	C	T	G	T
KJ522825	Kenya	T	-	-	-	-	A	-	-	-	-	G	C	T	G	T
KR014825	Zambia	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?

Specimen	Locality of origin	1889	1890	1892	1893	1894	1896	1897	2043	2044	2045	2046	2095	2096	2097	2113
ANO3.1	Bloemfontein Semi-Pristine Habitat 1	C	A	T	T	A	T	C	G	G	T	-	-	A	T	A
MK592071	Africa	T	T	C	A	C	T	T	A	G	T	T	A	A	C	T
KJ522825	Kenya	T	T	A	C	T	A	C	-	-	-	-	T	T	A	T
KR014825	Zambia	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?

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CHAPTER 4 – DISCUSSION

4.1 SAMPLING

Sampling efforts recovered a diverse array of mosquito species, belonging to taxa with distinct biological tendencies, host preferences and vectorial capacities (Jupp 1996). Many of these species serve as vectors of disease in South Africa, which include vectors of the Wesselsbron virus (*Ae. caballus*, *Ae. juppi*, *Ae. luridus*, *Ae. mcintoshi and Ae unidentatus*), WNV (*Cx. theileri* and *Cx. univittatus*), Sindbis (*Cx. univittatus*), RVF (*Ae. caballus*, *Ae*, *juppi*, *Ae. mcintoshi* and *Cx. theileri*), Middelburg virus (*Ae. caballus*, *Ae*, *juppi*) and incidental vectors of malaria (*An. squamosus*) (Gillies & De Meillon 1968, Jupp 1996, Hubálek *et al.* 2014, Braack *et al.* 2018) (Table 4.1).

The observed dominance of *Aedes* was a product of the genus' biology, since dormant drought-resistant eggs emerge in large numbers after rain, contributing to their sudden population surge (Jupp *et al.* 1980). A large portion of these *Aedes* specimens were collected at a Bloemfontein site during a period of heavier rainfall than usual (South African Weather Service 2022), which may have contributed to the surge in *Aedes* populations. Conversely, *Culex* mosquitoes tend to utilise more stable water sources for breeding, and are active throughout a large portion of the year (Jupp *et al.* 1980). This stability of *Culex* populations was reflected in this genus' steady presence across the numerous sampling sites. The current patterns were similarly recovered in the investigations of Jupp *et al.* (1980) and Van der Linde *et al.* (1982). Here, the studies of Jupp *et al.* (1980) and Van der Linde *et al.* (1982), conducted in the southern and central Free State, both spanned numerous years and produced substantial sample sizes (>20,000). These samples were significantly dominated by *Culex* rather than *Aedes*, while *Anopheles*, *Culiseta* and *Mansonia* formed a small portion of the collections, as recovered in the current study.

Since sampling was conducted across many localities in the Free State Province, the current study provided abundant distribution data for numerous species within the region. The generated data were also compared to the historical data documented by several publications (Table 3.3). Despite the widespread distribution of several sampled species in South Africa (*Ae. aegypti, Ae. dentatus, Ae. caballus, Cx. pipiens, Cx. quinquefasciatus, Cx. salisburiensis, Cx. theileri, Cx. univittatus* and *Cs. longiareolata*) (Jupp 1996), few publications have examined or documented the occurrence of these species within the Free State. Therefore, the generated distribution data of *Ae.* (*Stg.*) *aegypti* and *Ae.* (*Och.*) *juppi* extended north of the documented distribution in the Free State Province, thus supporting the ubiquitous occurrence of the *Ae.* (*Och.*) *juppi* in the region. On the other hand, several *Aedes* species exhibited ranges similar to the historical data, while two of these species (*Ae. caballus* and *Ae. mcintoshi*) were only sampled in a small portion of their documented range. The sampling results similarly expanded the range of *Cx.* (*Cux.*) *pipiens*, *Cx.* (*Cux.*) *quinquefasciatus*, *Cx.* (*Cux.*) *theileri* and *Cx.* (*Cux.*) *univittatus* towards the northern regions of the province, thus confirming the widespread occurrence of the latter three *Culex* species within the Free State Province. Finally, the occurrence of

Cx. (*Mai.*) *salisburiensis*, *An.* (*Cel.*) *cydippis* / *squamosus* and *Cs.* (*All.*) *longiareolata* was observed within the vicinity of their known distribution.

Table 4.1. Sampled species and their associated pathogens and host preferences.

SPECIES	VIRUS ISOLATION	EXPERIMENTAL TRANSMISSION OR INFECTION	POTENTIAL OR ESTABLISHED VECTOR	HOST AFFINITIES	REFERENCES
Ae. dentatus	ORUV, RVF, SFV, WSL	RVF	RVF	Humans, sheep, livestock	McIntosh 1972, Metselaar *et al.* 1974, McIntosh 1975, Tomori & Fabiyi 1975 in Tomori *et al.* 1976, Jupp *et al.* 1980, Van der Linde *et al.* 1982, Jupp & Cornel 1988
Ae. eritreae				Humans	McIntosh 1975
Ae. mixtus				Humans[1], livestock[1]	Bedford 1928 in Muspratt 1955, McIntosh 1975
Ae. fryeri	SPONV[2]				McIntosh *et al.* 1962
Ae. luridus	WSL[3] & MIDV[3]		WSL[4]	Humans, sheep, birds	McIntosh 1971, Jupp *et al.* 1980, Jupp 1996, Jupp & Kemp 1998
Ae. mcintoshi	MIDV, NRIV, RVF, WSL	RVF	RVF, WSL[4]	Humans, cattle, sheep, horses, chickens, birds[4]	McIntosh 1971 (*Ae. lineatopennis*), Jupp *et al.* 1980 (*Ae. lineatopennis*), Huang 1985, Jupp 1996, Van der Linde *et al.* 1982 (*Ae. lineatopennis*), Fontenille *et al.* 1998, Jupp & Kemp 1998
Ae. unidentatus		RVF	RVF, WSL[4]	Humans, sheep	McIntosh 1971, Jupp & Cornel 1988, Jupp 1996
Ae. caballus	MIDV[5], WSL[5], WNV	RVF	MIDV, RVF[5], WSL[5]	Humans, livestock, sheep[5], birds[5]	Muspratt 1955, McIntosh 1973, McIntosh *et al.* 1980, Jupp *et al.* 1980, Van der Linde *et al.* 1982, Jupp 1996, Jupp & Kemp 1998, Burt *et al.* 2002, Hubálek *et al.* 2014
Ae. juppi	MIDV[5], WSL[5]	RVF	RVF[5], WSL[5]	Humans, livestock, sheep[5], birds[5]	McIntosh 1973, McIntosh *et al.* 1980, Van der Linde *et al.* 1982, Jupp & Cornel 1988, Jupp 1996, Jupp & Kemp 1998
Ae. aegypti		BUNV, CHIKV, MIDV, PGAV, WNV, WSL	DENV, CHIKV, YFV	Humans	Belkin *et al.* 1970, Simasathien & Olson 1973, Metselaar *et al.* 1974, Huang 1979a, Boorman & Draper 1968, Baqar *et al.* 1993, Jupp 1996, Huang 2004
Ae. contiguus				Humans	Huang 2004
An. cydippis / squamosus			Malaria[6]	Humans[6], sheep[6], birds[6]	Gillies & De Meillon 1968, Jupp *et al.* 1980, Jupp & Kemp 1998
Cx. pipiens	NDUV			Sheep, birds	Jupp *et al.* 1980, Lutomiah *et al.* 2014
Cx. quinquefasciatus	USUV	WSL	Bancroftian filariasis, RVF	Birds	Bram 1967, Belkin *et al.* 1970, Simasathien & Olson 1973, Jupp *et al.* 1980, Van der Linde *et al.* 1982, Nikolay *et al.* 2011
Cx. theileri	WNV		RVF, WNV	Humans, sheep, birds	Jupp *et al.* 1980, Van der Linde *et al.* 1982, Jupp & Kemp 1998, Burt *et al.* 2002, Braack *et al.* 2018
Cx. univittatus	WNV		RVF, WNV, SINV	Sheep, birds	Jupp *et al.* 1980, Van der Linde *et al.* 1982, Jupp 1996, Burt *et al.* 2002, Nikolay *et al.* 2011
Cx. salisburiensis				Birds	Jupp *et al.* 1980
Cs. longiareolata				Sheep, birds	Jupp *et al.* 1980
Ma. africana	BANV, BUNV, CHIKV, PGAV				Boorman & Draper 1968, Metselaar *et al.* 1974

Banzi virus (BANV), Bunyamwera virus (BUNV), Chikungunya virus (CHIKV), Dengue virus (DENV), Middelburg virus (MIDV), Ndumu virus (NDUV), Ngari virus (NRIV), Orungo virus (ORUV), Pongola virus (PGAV), Rift Valley fever (RVF), Semliki Forest virus (SFV), Sindbis virus (SINV), Spondweni virus (SPONV), Usutu virus (USUV), Wesselsbron virus (WSL), West Nile virus (WNV), Yellow Fever virus (YFV).

1. Ae. *mixtus* / *microstictus*
2. Pool including *Ae. fryeri*
3. Pool including both *Ae. mcintoshi* and *Ae. luridus*
4. Refers to *Ae. mcintoshi* / *luridus* / *unidentatus*
5. *Ae. caballus* s.l.
6. Does not include *An. cydippis*

The prevalence of the most abundant sampled species (*Ae. dentatus*, *Ae. juppi*, *Cx. theileri*, *Cx. univittatus* and *Cx. pipiens*) was mainly supported by the results of the two other comparable studies in the region (Jupp *et al.* 1980 and Van der Linde *et al.* 1982). However, *Ae.* (*Adm.*) *dentatus* was relatively uncommon within their results (>1%), unlike the current findings. Both sets of authors recovered a similar proportion of *Ae.* (*Och.*) *caballus* and *Ae.* (*Neo.*) *mcintoshi*, a greater abundance of *An.* (*Cel.*) *squamosus* and a significantly smaller portion of *Cx.* (*Cux.*) *quinquefasciatus* than the current results.

Numerous other species were underrepresented within the province. This included *Ae.* (*Stg.*) *contiguus*, *Ae.* (*Coe.*) *fryeri*, *Ae.* (*Neo.*) *luteolateralis*, *Ae.* (*Cat.*) *mixtus*, *Cx.* (*Mai.*) *salisburiensis*, *Cx.* (*Cux.*) *simpsoni* and *Ma.* (*Mnd.*) *africana*. The scarcity of these species was also reflected by the results of Jupp *et al.* (1980) and Van der Linde *et al.* (1982). The authors sampled a combined total of more than 166,000 mosquitoes over several years, and collectively sampled a single *Ae.* (*Neo.*) *luteolateralis*, 60 *Cx.* (*Mai.*) *salisburiensis* and no *Ae.* (*Cat.*) *mixtus* specimens in the Free State Province. These samples included the first documented record of *Ae.* (*Neo.*) *luteolateralis* in the region (Van der Linde *et al.* 1982). The current study similarly yielded novel records for the Free State Province, including *Ae.* (*Adm.*) *eritreae*, *Ae.* (*Adm.*) *subdentatus*, *Ae.* (*Coe.*) *fryeri*, *Ae.* (*Stg.*) *contiguus*, *Cx.* (*Cux.*) *simpsoni* and *Ma.* (*Mnd.*) *africana*.

Although these species have not previously been observed within the Free State Province, they have still been recorded in South Africa. This included the occurrence of *Ae.* (*Adm.*) *eritreae*, *Ae.* (*Coe*) *fryeri*, *Ae.* (*Stg.*) *contiguus* and *Ma.* (*Mnd.*) *africana* in northeastern South Africa (Muspratt 1955, Worth & Paterson 1961, Jupp 1996, Gorsich *et al.* 2019, Guarido *et al.* 2021), *Ae.* (*Adm.*) *subdentatus* in the Eastern Cape, KwaZulu-Natal and Mpumalanga (Muspratt 1955, McIntosh 1975) and *Cx.* (*Cux.*) *simpsoni* in the North West, Gauteng, KwaZulu-Natal and Eastern Cape provinces (Muspratt 1955). These species were all documented within the vicinity of the Free State Province, since the listed records originated from adjacent provinces. Some distributions, such as those of *Ae.* (*Adm.*) *subdentatus* and *Cx.* (*Cux.*) *simpsoni* were also documented on two opposing sides of the Free State Province.

Due to the shortage of biodiversity studies within the province and the tendency of authors to focus on specific sampling localities, prior investigations likely only reflected the local species richness and population dynamics of mosquitoes. Therefore, the current study's sampling efforts provided a novel perspective on the mosquito diversity associated with numerous ecological units across the province. The presumed absence of several species may have been a product of their relative scarcity, or could have reflected their recent dispersal into new territories. Since many of the sampled species in the Free State are recognised vectors of disease, the introduction of novel species may have potential implications for the epidemiology of associated diseases.

Despite these species' relative scarcity, the use of several sampling methods contributed to the collection of multiple species. Other publications recovered similar associations between certain

species and specific sampling methods, as observed in the current results. This included the use of manual collections to sample *Ae.* (*Stg.*) *aegypti* (Morrison *et al.* 2004) and the collection of several species (*Ae. dentatus*, *Ae. eritreae*, *Ae. luteolateralis*, *Ae. subdentatus*, Ae. *unidentatus and Ma. africana*) with various CO_2 baited traps (Van der Linde *et al.* 1982, Logan *et al.* 1991, Guarido *et al.* 2021). However, the sampling efforts of other authors also revealed dissimilar associations, including *Cx.* (*Mai.*) *salisburiensis*, which has been collected with CO_2 baited New Jersey light and net traps (Van der Linde *et al.* 1982, Cornel *et al.* 2018) and *Ae.* (*Neo.*) *luridus,* which has been sampled with CO_2 baited CDC light traps (Sang *et al.* 2010). Authors also recovered *Cx.* (*Cux.*) *simpsoni* specimens with CDC and New Jersey light traps (Alahmed 2010) and *Ae.* (*Cat.*) *mixtus* with human landing catches (Diallo *et al.* 2019), while little information was available on the sampling of *Ae.* (*Stg.*) *contiguus*. Nonetheless, the CO_2 baited traps of numerous authors generally recovered a large number of species, which was also currently observed with the CO_2 baited net.

A few of the sites that were associated with a high species richness also had a moderate degree of anthropological disturbance. These sites were flanked by relatively intact natural grasslands, which might have served as a reservoir for the large mosquito diversity. Such natural habitats would provide mosquitoes with a wide array of potential hosts and food sources, thereby sustaining zoophilic or specialist feeders. Additionally, the anthropological influences likely facilitated mosquito breeding by providing continuous access to water, while reducing the presence of natural predators. This association between intermediate levels of disturbance and a greater species richness has also been observed in numerous other taxa, including trees, corals and ants (Connell 1978, Graham *et al.* 2009). This is known as the intermediate disturbance hypothesis, where disturbances sufficiently supress local communities to allow new species to colonise the region, without allowing sufficient time for competitive exclusion to occur (Connell 1978). These sites also generally entail a greater habitat heterogeneity (Graham *et al.* 2009), which may provide niches for a greater number of species. Although this effect was observed at several sampling localities, the interaction between pristine habitats and anthropological factors was not predictive of a high species richness across all sampling sites.

Several species also exhibited distinct associations with the various habitat type categories. The exclusive interaction of *Ae.* (*Stg.*) *aegypti* with urban habitats and *Ae.* (*Neo.*) *mcintoshi* with semi-pristine habitats may point to aspects of their documented biology. The tendency of *Ae.* (*Stg.*) *aegypti* to occur within urban habitats has been extensively documented, since its entire lifecycle can be completed within the vicinity of human activity (Morrison *et al.* 2008), where it preferentially feeds on humans (Scott *et al.* 1993). On the other hand, *Ae.* (*Neo.*) *mcintoshi* has been documented to feed on a wide range of hosts, including birds, ungulates and humans (Guarido *et al.* 2021), and therefore has the potential to inhabit a wide range of habitats. However, *Ae.* (*Neo.*) *mcintoshi* populations can fluctuate with rainfall (Guarido *et al.* 2021), and unfavourable conditions may have affected the species' presence across the various sampling sites.

Numerous factors contributed to the successful collection of diverse mosquito species in the region. These species subsequently served as a foundation for the first molecular representations of *Ae*. (*Stg*.) *contiguus*, *Ae*. (*Neo*.) *luridus*, *Ae*. (*Neo*.) *luteolateralis*, *Ae*. (*Cat*.) *mixtus* and *Cx*. (*Mai*.) *salisburiensis* for the COI region; *Ae*. (*Adm*.) *dentatus*, *Ae*. (*Och*.) *juppi*, *Ae*. (*Neo*.) *luridus* and *Cx*. (*Cux*.) *univittatus* for the ITS2 region; and *Ae*. (*Adm*.) *dentatus*, *Ae*. (*Coe*.) *fryeri*, *Ae*. (*Och*.) *juppi* and *Cx*. (*Cux*.) *univittatus* for the 28S region, which were deposited in the BOLD database.

4.2 MULTIGENE APPROACH AND TOPOLOGICAL INCONGRUENCES

Incongruences between different phylogenetic methods and gene regions have been well-documented by numerous authors (Baker *et al*. 1997, Sota & Vogler 2001, Jeffroy *et al*. 2006, Sung *et al*. 2007). Some of these discrepancies may be the product of different modes of inheritance (Moore 1995), varying degrees of substitutional saturation and different evolutionary rates (Baker *et al*. 1997, Sung *et al*. 2007). Since mitochondrial and nuclear DNA are subject to different modes of inheritance, they can produce different phylogenetic results (Moore 1995, Sota & Vogler 2001), yet authors have reached conflicting conclusions on the applicability of such gene regions. Moore (1995) concluded that mitochondrial phylogenies are more likely to be congruent with the evolutionary history of a taxon, while the analyses of Sota and Vogler (2001) generally recovered a greater congruence between the nuclear phylogenies of carabid beetles and their morphologically defined taxonomic units. However, Reidenbach *et al.* (2009) recovered incongruences among the phylogenetic topologies of mosquito species based on six nuclear genes, especially involving intergeneric relationships.

In addition to incongruences between gene regions, there may also be discordance between the phylogenetic results and the true evolutionary history of a lineage (Sota & Vogler 2001). This may be caused by substitutional saturation, horizontal gene transfer, introgression and incomplete lineage sorting (Bull *et al*. 1993, Advise 2004, Engstrom *et al*. 2004). Substitutional saturation can result in homoplastic characters that obscure deeper relationships between taxa, as observed in protein-coding genes (Baker *et al*. 1997, Engstrom *et al*. 2004, Sung *et al*. 2007). With horizontal gene transfer, a portion of one species' genetic material becomes incorporated within another species, resulting in a genome with heterogeneous histories and varying affiliations towards the 'donor' genome (Bull *et al*. 1993). Hybridisation facilitates introgression, a relatively common occurrence that ranges from sterile F1 hybrids to the frequent gene exchange between taxa (Advise 2004). Finally, discrepancies may be caused by incomplete lineage sorting, where the genealogy of alleles is not correlated with the species tree due to the independent inheritance of alleles in each daughter species (Advise 2004).

Therefore, another approach attempts to incorporate the data from multiple genes to investigate the phylogeny of a group, which improves the phylogenetic resolution, clade support and the utility of phylogenetic results (Devulder *et al*. 2005). This multigene approach has also been employed on a small scale to elucidate culicid relationships and lineages (Puslednik *et al*. 2012, Greni *et al*. 2018). The

use of the multigene approach is advantageous, since the compounded residual signal from each gene and the unique information provided by each DNA region contribute to the resolution and support of the overall phylogeny, despite incongruences among trees generated from single genes (Baker *et al*. 1997, Mitchell *et al*. 2000, Sung *et al*. 2007).

Within phylogenetic investigations, topologies may also be sensitive to taxonomic coverage, where an increased number of characters can contribute to clade support (Braun & Kimball 2002, Sung *et al*. 2007). Here, the multitude of characters afforded by the multigene approach tends to yield both higher support values and a greater number of significantly supported clades (Mitchell *et al*. 2000). Therefore, the ideal dataset consists of multiple DNA regions, represented by a common set of specimens spanning the various genes. Unfortunately, very few mosquito specimens within public DNA databases are represented by multiple gene regions. Therefore, the use of such a limited concatenated dataset would not yield any meaningful insight into the numerous intrageneric divisions of mosquitoes.

The current study's focus was rather to ensure that species remained as consistent as possible across the various single-gene datasets. Since fewer sequences were available for the ITS2 and 28S DNA regions, some species were not included in all analyses. The exclusion of certain taxa from specific analyses impacted the final topology of the results. However, numerous common features were still observed across multiple DNA regions. This included the general affiliations between clusters of taxa, the well-supported associations between closely related species and the non-monophyly of numerous subgenera and divisions. This shared phylogenetic signal likely reflected the underlying evolutionary history of these taxa, thus serving as a foundation for the systematic and taxonomic inferences.

4.3 PHYLOGENETIC DISCUSSION

Many of the current phylogenetic findings were corroborated by multiple DNA regions, which consisted of recurring affiliations and numerous non-monophyletic taxa. Since the scope of the current study was centred on the phylogeny of South African mosquitoes, the discussion has been restricted to relevant species and taxonomic divisions. Additionally, this section primarily focuses on results supported by multiple analyses. Here, morphological, biogeographical and phylogenetic findings are incorporated to provide context to the phylogenetic groupings and systematic challenges unearthed by the current results.

4.3.1 *AEDES*

Subgenera *Aedes* + *Neomelaniconion*

In the current analyses (COI and ITS2), the subgenus *Neomelaniconion* was consistently situated in close proximity to the subgenus *Aedes* (Fig. 3.7 & 3.8), sharing a moderately supported clade in the COI Bayesian results (PP 94, value not shown). This affiliation was also recovered by the findings of

other authors, including Reinert *et al*. (2009) and Somboon *et al*. (2021). In the COI ML analysis of Somboon *et al*. (2021), the authors recovered an unsupported clade consisting of *Aedes*, *Neomelaniconion* and several other taxa. Their Bayesian analysis also produced an unsupported clade that included *Aedes* and *Neomelaniconion*, which individually shared a polytomy with other subgenera and the genus *Verrallina*. Reinert *et al*. (2009) similarly examined the phylogeny of Aedini based on morphological data, where their analysis recovered an unsupported clade that included *Neomelaniconion*, *Aedes* and several other taxa.

This morphological dataset of Reinert *et al*. (2009) included many larval, pupal and adult characteristics that were shared between *Aedes* and *Neomelaniconion*. However, these features were also present within numerous other taxa. Some of the most exclusive shared traits included the larval 8-P to 4-P setal length ratio, the unbranched larval 6-III seta, the subapical attachment of the gonostylus and the distally widened aedeagus with its teeth restricted to the distal half. Additionally, both subgenera shared the tendency to oviposit in naturally occurring freshwater sources. Despite their shared characteristics, these subgenera have different distributions, where *Aedes* predominantly occurs in the Palaearctic Region and *Neomelaniconion* primarily within the Afrotropical Region (Mosquito Taxonomic Inventory 2021). Considering their abutting distributions and shared morphological and molecular features, these subgenera may have a relatively obscured shared ancestry, since neither morphological nor molecular analyses could produce a well-supported relationship between *Aedes* and *Neomelaniconion*.

Affiliations of sampled *Neomelaniconion* species (*Ae*. *luridus*, *Ae*. *luteolateralis*, *Ae*. *mcintoshi* and *Ae*. *unidentatus*)

The affiliations between the various *Neomelaniconion* species were either inconsistent or represented by a single dataset, yet all analyses produced a non-monophyletic *Ae*. (*Neo*.) *mcintoshi*. In the COI analyses (Fig. 3.7), the sampled and non-sampled *Ae*. (*Neo*.) *mcintoshi* specimens were intermixed with many other species within the subgenus and were not stratified according to their geography. However, the ITS2 analyses (Fig. 3.8) produced separate clusters of South African and Kenyan *Ae*. (*Neo*.) *mcintoshi* lineages. In both datasets, the identity of one *Ae*. (*Neo*.) *mcintoshi* specimen was ambiguous due to the presence of light coloured sterna (Fig. 3.6 c; AED20.405). This morphological deviation was not associated with its phylogenetic placement, since the specimen grouped closely with another sampled *Ae*. (*Neo*.) *mcintoshi* specimen in the ITS2 results and was one of the many scattered *Ae*. (*Neo*.) *mcintoshi* specimens in the COI results.

Within the COI haplotypes of *Ae*. (*Neo*.) *mcintoshi* (Table 3.9), no alleles differentiated South African specimens from other African sequences, since many alleles were sporadically shared between populations. The three Bloemfontein specimens also individually possessed numerous unique SNPs, and their haplotypes were not clearly stratified by their geographical origins. On the other hand, the ITS2 Kenyan sequences differed significantly from the sampled Bloemfontein specimens (Table 3.10), possessing numerous distinct alleles and indels. This may have been a product of the relatively unconstrained evolution of the ITS2 region, compared to the protein-coding COI gene. Furthermore,

the unique morphology of specimen AED20.405 was not associated with a markedly distinct haplotype in any of the datasets, which was also reflected in the phylogenetic results.

To date, only a few other phylogenetic studies have focused on the subgenus *Neomelaniconion*, thereby complicating any interpretations on the affiliations of its members. However, Ajamma *et al*. (2016) conducted a COI ML analysis, which recovered a non-monophyletic *Ae*. (*Neo.*) *mcintoshi* that was associated with *Ae*. (*Neo.*) *circumluteolus*, similar to the results of the present study. The COI ML analysis of Guarido *et al*. (2021) likewise recovered a non-monophyletic *Ae*. (*Neo.*) *mcintoshi* associated with *Ae*. (*Neo.*) *circumluteolus* and another *Neomelaniconion* species. This non-monophyly of *Ae*. (*Neo.*) *mcintoshi* may have been the by-product of its widespread distribution in the Afrotropical Region (Zavortink 1990) and the genetic variation accompanying these geographically distant populations.

Despite the morphological similarity of the two sampled specimens, *Ae*. (*Neo.*) *unidentatus* was also non-monophyletic in the current study's COI analyses (Fig. 3.7). The specimens originated from two different localities within the Free State Province. Here, these specimens were either associated with *Ae*. (*Neo.*) *luridus* or *Ae*. (*Neo.*) *mcintoshi*, where the latter affiliation was also recovered in the COI ML analysis of Guarido *et al*. (2021). In the ITS2 results, *Ae*. (*Neo.*) *luridus* was most closely associated with *Ae*. (*Neo.*) *circumluteolus* (Fig. 3.8). However, *Ae*. (*Neo.*) *unidentatus* was not included in the ITS2 dataset and its affiliations could not be confirmed.

The non-monophyly of these *Neomelaniconion* species could potentially reflect the phylogenetic limitations of these DNA regions. However, Zavortink (1990) has demonstrated the potential for gene flow between these taxa, and such instances of introgression could also obscure these interspecific relationships. The forced matings by Zavortink (1990) successfully produced fertile hybrids from crosses between *Ae*. (*Neo.*) *mcintoshi* x *Ae*. (*Neo.*) *circumluteolus*, *Ae*. (*Neo.*) *mcintoshi* x *Ae*. (*Neo.*) *luteolateralis* and *Ae*. (*Neo.*) *unidentatus* x *Ae*. (*Neo.*) *mcintoshi*, which demonstrated a lack of postzygotic barriers between these species. Zavortink (1990) additionally observed field specimens that were similar but not identical to *Ae*. (*Neo.*) *mcintoshi*. These results, coupled with the present study's non-monophyly of several species and the prominent variation observed in the sternal colouration of *Ae*. (*Neo.*) *mcintoshi* specimens, produced doubts regarding the status of several *Neomelaniconion* species.

Zavortink (1990) also provided the only available publication that illustrated the internal phylogenetic structure of *Neomelaniconion*, based on morphological and electrophoretic data. The various analyses generally produced incongruent topologies, while a few features of a maximum parsimony analysis based on equally-weighted adult data resembled aspects of the current results. Here, the author recovered a sister relationship between *Ae*. (*Neo.*) *luteolateralis* and *Ae*. (*Neo.*) *mcintoshi*, which was collectively sister to *Ae*. (*Neo.*) *unidentatus*, followed by *Ae*. (*Neo.*) *luridus* and finally *Ae*. (*Neo.*) *circumluteolus*. The pairing of *Ae*. (*Neo.*) *luteolateralis* + *Ae*. (*Neo.*) *mcintoshi* and the relatively close

relationship between Ae. (*Neo.*) *unidentatus* and *Ae.* (*Neo.*) *luridus* were similar to the current study's COI results, while the affiliation between *Ae.* (*Neo.*) *luridus* and *Ae.* (*Neo.*) *circumluteolus* was recovered in the current ITS2 results. However, Zavortink (1990) noted that numerous morphological characteristics of *Neomelaniconion* are generally not phylogenetically informative, and phylogenetic analyses based on such characteristics may not bear much weight. Since the internal relationships of South African *Neomelaniconion* species have not previously been examined from a molecular perspective, the potential for hidden affiliations within the subgenus remains. The non-monophyly of several *Neomelaniconion* species might warrant further investigations to either confirm the presence of naturally occurring hybrids, or examine the potential for cryptic species within the subgenus.

Subgenera *Aedimorphus* + *Stegomyia*

Although specific affiliations were inconsistent between analyses, *Aedimorphus* was consistently recovered as polyphyletic and frequently associated with *Stegomyia*, where both subgenera were exclusively represented by South African species. Several studies also investigated the phylogenetic relationships of *Aedimorphus* and *Stegomyia*, which recovered similar results. This included the COI nucleotide sequence analysis of Ajamma *et al.* (2016), where *Aedimorphus* was polyphyletic due to the separate placement of *Ae.* (*Adm.*) *dentatus*. Here, *Ae.* (*Adm.*) *dentatus* was associated with *Neomelaniconion*, while the main *Aedimorphus* grouping was sister to a non-monophyletic *Stegomyia*, with a low degree of support. Ajamma *et al.*'s (2016) ITS2 nucleotide sequence analysis similarly produced a non-monophyletic *Stegomyia* with the inclusion of *Aedimorphus*. In the analyses of Shepard *et al.* (2006) based on the secondary structure alignment of the 18S sequences, *Stegomyia* was likewise rendered paraphyletic by *Aedimorphus*. The ML COI sequence analysis of Cook *et al.* (2005) yielded a weakly supported sister relationship between *Stegomyia* and *Aedimorphus*, while *Diceromyia* was situated medially between *Aedimorphus* and *Stegomyia* in the COII analysis. Isoe (2000) also recovered a close relationship between *Aedimorphus* and *Stegomyia* based on vitellogenin gene sequences.

On the other hand, the morphological analysis of Reinert *et al.* (2009) produced a weakly supported monophyletic *Aedimorphus*, as defined by the traditional systematic framework. Since the basal relationships were generally unsupported, the authors noted that the monophyletic status of *Aedimorphus* still needs to be confirmed. Nonetheless, Wilkerson *et al.*'s (2015) reanalysis of Reinert *et al.*'s (2009) data once again produced a weakly supported paraphyletic *Aedimorphus*. The morphological dataset of Reinert *et al.* (2009) included numerous immature and adult characteristics that were shared between *Aedimorphus* and *Stegomyia*. These features were also shared with numerous other taxa, where the most exclusive characters included the egg shape and its well-developed cell pattern. The subgeneric key of Jupp (1996) also listed the absence of mesanepimeral setae as a common characteristic, while the key of Edwards (1941) listed several shared male characteristics, including genital and palpal features. In addition to these shared morphological features, the subgenera also share their distribution in the Afrotropical, Oriental and Australian Regions (Mosquito Taxonomic Inventory 2021).

This weak association between *Aedimorphus* and *Stegomyia* has been recovered by several authors and supported within multiple current analyses. It seems likely that both subgenera are non-monophyletic assemblages, which becomes apparent with more extensive taxonomic coverages. Since the affiliation between these taxa was generally unsupported, it may reflect a residual phylogenetic association obscured by substitutional saturation.

Affiliations of sampled *Aedimorphus* species (*Ae. dentatus* & *Ae. eritreae*)

Aedes (*Adm.*) *dentatus* was represented by a single sampled specimen in the ITS2 (Fig. 3.8) and 28S (Fig. 3.9) datasets, where its placements and affiliations were inconsistent between analyses. In the ITS2 results, this species was included in an unsupported clade consisting of *Ae.* (*Stg.*) *albopictus*, *Ae.* (*Adm.*) *vexans* and *Ae.* (*Pmt.*) *albocephalus*. Here, *Ae.* (*Adm.*) *dentatus* was most closely affiliated with *Ae.* (*Stg.*) *albopictus*, which was well-supported in the Bayesian analysis (PP 98). However, its placement differed in the 28S results, where it was most closely associated with *Ae.* (*Coe.*) *fryeri* in an unsupported clade.

Aedes (*Adm.*) *dentatus* was not represented in many publications, yet was included in the phylogenetic investigations of two separate authors. The COI sequence analysis of Ajamma *et al.* (2016) produced unique results where *Ae.* (*Adm.*) *dentatus* was placed within a well-supported clade of *Neomelaniconion*. However, the morphological analysis of Reinert *et al.* (2009) placed the species within a clade of *Aedimorphus*, where it was situated medially between the *Ae.* (*Adm.*) *vexans* and *Ae.* (*Adm.*) *gibbinsi* + *Ae* (*Adm.*) *quasiunivittatus*. Within Reinert *et al.*'s (2009) morphological dataset, *Ae.* (*Adm.*) *dentatus* possessed numerous unique characteristics that distinguished it from other *Aedimorphus* species. It was the only *Aedimorphus* species that possessed cercal scales, a relatively common characteristic in *Stegomyia*. Nonetheless, no consistent affiliations were recovered between the current study's results and those recovered within other publications, and therefore no definitive conclusions could be made regarding *Ae.* (*Adm.*) *dentatus'* affiliations.

Aedes (*Adm.*) *eritreae* was also represented by a single sampled specimen in the COI analyses (Fig. 3.7). This species was included within a clade of *Ae.* (*Cat.*) *tarsalis* + *Ae.* (*Adm.*) *leesoni*, which was moderately supported in the Bayesian analysis (PP 93, value not shown). However, this species was not well-represented within the literature and was only included in the results of two publications. In the morphological analysis of Reinert *et al.* (2009), *Ae.* (*Adm.*) *eritreae* was situated among other members of the same subgenus, *Aedimorphus*, where it formed a well-supported grouping with *Ae.* (*Adm.*) *dalzieli*. *Aedes* (*Adm.*) *eritreae* was also situated relatively close to *Ae.* (*Adm.*) *cumminsii* within the COI ML analysis of Guarido *et al.* (2021). Therefore, both the current study and the literature's results support this species' affiliation with *Aedimorphus*.

Affiliations of sampled *Coetzeemyia* species (*Ae. fryeri*)

Aedes (*Coe.*) *fryeri* was represented by a single sampled specimen in the 28S analyses (Fig. 3.7), where it was associated with *Ae.* (*Adm.*) *dentatus* (PP 85; BS 67), while additional affiliations were inconsistent. Few comparable findings were available, since *Coetzeemyia* was underrepresented within the phylogenetic literature, likely due to its monotypic nature and recent establishment. Jonathan (2020) nonetheless examined the phylogeny of *Ae.* (*Coe.*) *fryeri*, based on the mitochondrial nicotinamide adenine dinucleotide hydride dehydrogenase subunit 4 (ND4) sequences. Here, this species formed a well-supported clade with *Ae.* (*Zav.*) *fulgens*, which was in turn related to *Ae.* (*Adm.*) *tricholabis*. *Coetzeemyia*'s close association with *Aedimorphus* was also recovered in the current study's results, yet it could not be confirmed by other DNA regions. Due to the subgenus' underrepresentation within the literature and molecular databases, further analyses are required to clarify its placement and affiliations within the genus *Aedes*.

Subgenus *Stegomyia*

Stegomyia was recovered as a polyphyletic assemblage in the COI and ITS2 analyses (Fig. 3.7 & 3.8), which consisted of a relatively large number of *Stegomyia* species. Multiple analyses also recovered the well-supported monophyly of the Simpsoni Group and its association with the Metallicus Group. Additionally, these two groups formed a well-supported clade with the Poweri Group in the COI analyses (PP 100; BS 81) (Fig. 3.7). However, *Stegomyia* was only represented by two species in the 28S analyses (Fig. 3.9), where it formed an unsupported monophyletic clade due to the low taxonomic coverage.

The monophyly of *Stegomyia* was similarly inconsistent within the available literature. On the one hand, several authors recovered a non-monophyletic *Stegomyia*, including Isoe (2000), Reinert *et al.* (2004), Shepard *et al.* (2006) and Ajamma *et al.* (2016). The COI and ITS2 nucleotide sequence analyses of Ajamma *et al.* (2016) produced a non-monophyletic *Stegomyia*, which incorporated *Fredwardsius* and *Catageiomyia* in the COI results, and *Ae.* (*Adm.*) *hirsutus* in the ITS2 results. The analyses of Shepard *et al.* (2006), based on the secondary structure alignment of the 18S sequences, also placed an *Aedimorphus* species within a *Stegomyia* clade. Finally, the multiple analyses of Isoe (2000) based on the vitellogenin gene sequences produced *Stegomyia* clades that included *Armigeres subalbatus* (Coquillett, 1898), while the implied weight and equal weight morphological analyses of Reinert *et al.* (2004) based on adult data produced a polyphyletic *Stegomyia*.

Conversely, several authors recovered *Stegomyia* as a monophyletic assemblage. This included the COI and COII sequence analyses of Cook *et al.* (2005), where only three species were included in the dataset. The combined adult and immature data analyses of Reinert *et al.* (2004) similarly produced a monophyletic *Stegomyia*, which only included five specimens. This monophyly was again recovered in the subsequent results of Reinert *et al.* (2009), where the morphological analysis included 28 *Stegomyia* species and produced an unsupported but monophyletic subgenus.

The results of Reinert *et al*. (2009) produced two prominent *Stegomyia* branches, where one included the Aegypti, Metallicus, Poweri and Simpsoni Groups, while the other included the Scutellaris and Unilineatus Groups. The affiliations between the Simpsoni, Metallicus and Poweri Groups reflected the current study's COI results (Fig. 3.7), while the Simpsoni, Metallicus and Aegypti Group relationships were similar to the current ITS2 results (Fig. 3.8). Within the morphological dataset of Reinert *et al*. (2009), numerous characteristics differentiated the Aegypti / Metallicus / Poweri / Simpsoni Group species from the Scutellaris / Unilineatus Groups. This included features of the pupal 7-CT seta and paddle fringe, in addition to numerous male genital characteristics. Jupp (1996) also distinguished the Aegypti / Metallicus / Poweri / Simpsoni Groups by the scutum's white anterolateral markings and the distally narrowed gonostylus. In addition to their shared morphology and phylogenetic affiliations, the Aegypti / Metallicus / Poweri / Simpsoni Groups also share an Afrotropical distribution (Mosquito Taxonomic Inventory 2021), providing support for their common evolutionary origin.

Polyphyletic non-sampled South African *Stegomyia* species (*Ae. bromeliae* & *Ae. simpsoni*)

Multiple analyses recovered the polyphyletic and intermixed topology of *Ae.* (*Stg.*) *bromeliae* and *Ae.* (*Stg.*) *simpsoni* (Fig. 3.12). Within the 11 COI polymorphic sites, only a single allele was associated with the assigned species identities, while no discerning allele was present in the ITS2 haplotypes. The species' close relationship is exemplified by their taxonomic history, since *Ae.* (*Stg.*) *bromeliae* was initially treated as a synonym of *Ae.* (*Stg.*) *simpsoni* (Edwards 1932) and later resurrected from synonymy (Huang 1979b). Despite the morphological similarity of these species, specimens can be distinguished by the structure of their tarsal claws and the patterning of their tarsomeres (Huang 1979b). Therefore, the discordance between their species identities and the phylogenetic placements might reflect phylogenetic or taxonomic challenges. However, since no other relevant phylogenetic studies could be located, inferences on the taxonomic status of these species are limited.

Affiliations of sampled *Stegomyia* species (*Ae. aegypti* & *Ae. contiguus*)

Within the COI analyses (Fig. 3.13), the sampled *Ae.* (*Stg.*) *aegypti* specimens congregated with their conspecifics and showed no apparent distinction between sampled and non-sampled specimens, thereby supporting the specimens' identity. The haplotypes of the two sampled specimens uniquely shared alleles with different populations of non-South African sequences (Table 3.5), which consisted of either Chilean specimens or a sequence derived from a laboratory colony specimen (Liverpool strain). These two sampled specimens were morphologically similar, yet originated from two different regions in the Free State Province. They displayed numerous sequence differences in the COI region, which may have been the product of population-level or individual differences. Their phylogenetic affinities were reflected by the haplotype structure, where the *Ae.* (*Stg.*) *aegypti* groupings were not associated with their geography. This incongruity between the specimens' origin and their phylogenetic groupings may reflect the dispersal capabilities of the species, since it is closely associated with anthropological

activity (Scott *et al.* 1993, Morrison *et al.* 2008) and has inadvertently been transported to numerous novel geographical regions (Belkin *et al.* 1970, Huang 1979a). Therefore, the introduction of geographically distinct individuals would contribute novel haplotypes to the population. Furthermore, this discrepancy may also point to aspects of *Ae.* (*Stg.*) *aegypti'*s evolutionary history. The phylogenetic analysis of Moore *et al.* (2013), based on African ND4 haplotypes, also recovered *Ae.* (*Stg.*) *aegypti* clades that were unrelated to their morphology or geographic origin, and the authors stated that it supported the well-documented theory that the species as a whole may have originated from two separate lineages.

However, the interspecific affiliations of *Ae.* (*Stg.*) *aegypti* were either unsupported or inconsistent within the current analyses, as observed in other phylogenetic studies (Shepard *et al.* 2006, Sota & Mogi 2006, Reinert *et al.* 2009, Ajamma *et al.* 2016). The COI sequence analysis of Ajamma *et al.* (2016) recovered a weakly supported sister relationship between *Ae.* (*Stg.*) *aegypti* and *Catageiomyia* + *Fredwardsius* + *Stegomyia* (Africanus & Metallicus Groups), while the ITS2 dataset produced a relationship between *Ae.* (*Stg.*) *aegypti* and *Aedimorphus* + *Stegomyia* (Africanus & Metallicus Groups). The neighbour-joining (NJ) and ML bootstrap analyses of Shepard *et al.* (2006), based on the secondary structure alignment of the 18S sequences, produced a well-supported clade consisting of *Ae.* (*Stg.*) *aegypti* (Aegypti Group), *Ae.* (*Stg.*) *albopictus* (Scutellaris Group) and *Ae.* (*Adm.*) *vexans*. Within the morphological analysis of Reinert *et al.* (2009), *Ae.* (*Stg.*) *aegypti* was sister to *Ae.* (*Stg.*) *apicoargentea* (Apicoargenteus Group) and closely related to the Poweri + Simpsoni Groups. However, the authors noted that the relationship between *Ae.* (*Stg.*) *aegypti* and *Ae.* (*Stg.*) *apicoargentea* was not substantiated by their morphology. Lastly, the combined COI, 16S and 28S sequence analysis of Sota and Mogi (2006) produced a well-supported sister relationship between *Ae.* (*Stg.*) *aegypti* and *Ae.* (*Stg.*) *wadai* (Pseudonigeria Group), with no close affiliation to *Ae.* (*Stg.*) *albopictus*.

Unlike *Ae.* (*Stg.*) *aegypti*, the second sampled *Stegomyia* species (*Ae. contiguus*, Poweri Group) was underrepresented in the current analyses. Limited sequence data was available for *Ae.* (*Stg.*) *contiguus*, since a single specimen was successfully sequenced for the COI region (Fig. 3.7) and no database sequences were available for this species. Nonetheless, this specimen's close affiliation with *Ae.* (*Stg.*) *metallicus* was well-supported (PP 100; BS 88), which could not be substantiated by other analyses.

Subgenus Ochlerotatus

Ochlerotatus was ubiquitously non-monophyletic in all current analyses, and its interspecific relationships were either inconsistent or unsupported. However, multiple analyses recovered *Ochlerotatus*' affinity towards *Jarnellius*, *Downsiomyia* and *Dobrotworskyius*, which was generally weakly supported. Other studies similarly recovered a polyphyletic *Ochlerotatus*, although its affiliations mainly differed from the current study's results. Since the scope of species included within *Ochlerotatus* has shifted dramatically within the last few decades, the current discussion refers to the subgenus as defined by the traditional systematic framework of Aedini. The morphological analysis of Reinert *et al.*

(2006) recovered a paraphyletic *Ochlerotatus* clade with the inclusion of *Rusticoidus*. *Ochlerotatus* was also paraphyletic in the implied weight morphological analysis of Reinert *et al*. (2008), with the inclusion of *Levua*, *Geoskusea*, *Sallumia*, *Rhinoskusea* and *Rusticoidus*. Therefore, Reinert *et al*. (2008) recognised, erected or reinstated these taxa as subgenera of *Ochlerotatus*. However, their analysis did not produce a close relationship between *Ochlerotatus* and *Dobrotworskyius*, *Downsiomyia* or *Jarnellius*, as recovered in the current study's results. In the ML COI sequence analysis of Cook *et al*. (2005), *Ochlerotatus* was also paraphyletic and included *Acartomyia* and *Dahliana* in the unsupported clade.

Nonetheless, three studies produced results that reflected the affiliations recovered in the current study. This included the COI sequence analyses of Somboon *et al*. (2021), which produced a polyphyletic *Ochlerotatus*. Although these clades were not well-supported, the Woodius Group of *Ochlerotatus* shared a clade with *Dobrotworskyius*, *Downsiomyia* and several other subgenera in both the Bayesian and ML analyses. *Ochlerotatus* was also non-monophyletic in the NJ COI sequence analysis of Batovska *et al*. (2016) and was included in an unsupported clade with *Dobrotworskyius* and two other subgenera. However, the sister relationship between *Dobrotworskyius* and *Ae*. (*Och*.) *camptorhynchus* was well-supported (BS 88). Lastly, the morphological analysis of Reinert *et al*. (2009) also produced a non-monophyletic *Ochlerotatus* with the inclusion of two other subgenera. Here, *Jarnellius* was situated basally and in close proximity to *Ochlerotatus*, while *Downsiomyia* and *Dobrotworskyius* were not closely related. These subgenera also occur in different geographic regions, yet portions of their distribution still overlap (Mosquito Taxonomic Inventory 2021). The species diversity of *Ochlerotatus* is mainly centred in the Nearctic, Neotropical and Australian Regions, which overlaps with the Nearctic distribution of *Jarnellius* and the Australian distribution of *Dobrotworskyius*. On the other hand, *Downsiomyia* has a relatively unique distribution in the Oriental Region. Despite the recovered association between these subgenera, the inconsistent and unsupported affiliations resulted in inconclusive results, therefore limiting any inferences regarding their shared evolutionary history.

Affiliations of sampled *Ochlerotatus* species (*Ae*. *caballus* & *Ae*. *juppi*)

The sampled individuals of the Juppius Group, *Ae*. (*Och*.) *caballus* and *Ae*. (*Och*.) *juppi*, formed distinct clades within the phylogenetic results, which supported their assigned species identities. Both *Ae*. (*Och*.) *caballus* and *Ae*. (*Och*.) *juppi* were also mutually affiliated in multiple analyses, where sampled individuals jointly formed well-supported groupings in the COI and ITS2 results (Fig. 3.7 & 3.8). However, these species were underrepresented within other phylogenetic studies. Here, only the morphological analysis of Reinert *et al*. (2009) placed *Ae*. (*Och*.) *caballus* as a sister clade to *Ae*. (*Och*.) *spilotus* (Stricklandi Group), with a moderate degree of support (GC 38). This grouping was situated within a larger non-monophyletic *Ochlerotatus* clade with a relatively close relationship to *Rusticoidus*. Conversely, no phylogenetic comparisons were available for *Ae*. (*Och*.) *juppi*. Despite the lack of comparable phylogenetic studies, the common morphological features of these species substantiate their mutual affiliation. Huang and Rueda (2014) listed the dark coloured proboscis and the presence

of white scales on the scutellum and subspiracular area as shared characteristics between these Afrotropical *Ochlerotatus* species, while Jupp (1996) additionally listed the basal tarsomeral banding as a shared feature. These species also occur within the Afrotropical Region (Mosquito Taxonomic Inventory 2021) and share a large portion of their range within South Africa (Jupp 1996).

Within the current datasets, the South African and Iranian *Ae.* (*Och.*) *caballus* specimens were genetically distinct from one another (Table 3.6 & 3.7). Numerous alleles and indels were unique to the two populations and consistently resulted in separate lineages within the phylogenetic results, leading to the species' polyphyletic structure in the COI results (Fig. 3.7). Since the two populations were geographically distinct, such unique genetic profiles could be expected. Furthermore, numerous unique SNPs were observed within the sampled Bloemfontein specimens, yet the morphologically divergent male specimen with LMS (AED18.4) did not have a markedly unique haplotype. This specimen is likewise grouped securely with morphologically typical specimens in the phylogenetic results. The second species, *Ae.* (*Och.*) *juppi* was exclusively represented by sampled individuals. These specimens were phylogenetically homogenous, despite the presence of numerous unique SNPs within the COI dataset (Table 3.8), which seemed to occur independent of their morphology and sampling locality.

Therefore, all South African specimens of the Juppi Group (*Ae. caballus* & *Ae. juppi*) showed a close mutual affiliation, where internal groupings were not associated with their morphology or biogeography. However, the inconsistent affiliation between South African and Iranian *Ae.* (*Och.*) *caballus* specimens may warrant further investigation into the specific status of such geographically distinct populations, especially since no other geographical populations of *Ae.* (*Och.*) *caballus* were represented by the target DNA regions at the time of analysis.

Affiliations of sampled *Catageiomyia* species (*Ae. mixtus*)

Aedes (*Cat.*) *mixtus* was the only sampled species of *Catageiomyia* and was included within the COI analyses (Fig. 3.7), where it had a well-supported affiliation with *Ae.* (*Cat.*) *argenteopunctatus* (PP 100; BS 97). This affiliation between the two *Catageiomyia* species is also substantiated by their shared morphology, where the key of Jupp (1996) included colouration, broad whitish scales on the scutellum, broad decumbent scales on the vertex and numerous male genital characteristics as shared features between these species. Both species also share a common distribution in South Africa and Zimbabwe (Jupp 1996). However, the affiliations of *Ae.* (*Cat.*) *mixtus* could not be substantiated due to a lack of other comparable phylogenetic studies.

4.3.2 *CULEX*

Subgenus *Culex*

The subgenus *Culex* was represented by numerous species in the current analyses, where it was consistently recovered as a non-monophyletic assemblage. Here, the subgenera *Barraudius* and *Oculeomyia* were often incorporated within the overarching clade and *Oculeomyia* was often situated proximally to the Sitiens Group. The subgenus *Culex* was also recovered as a non-monophyletic group in other publications, including the morphological analyses of Harbach *et al*. (2012), where the subgenus *Culex* was intermixed with several other subgenera. Based on the complete datasets (K = 9 & K = 16), their analyses produced a relatively close but unsupported relationship between the subgenus *Culex* (Atriceps Group) and *Barraudius*. *Oculeomyia* also had a close but unsupported relationship with members of the Sitiens Group. The morphological analyses of Harbach *et al*. (2017) once again produced a close relationship between the subgenera *Barraudius* and *Culex*, which was retained in the collapsed tree showing only positive GC values. Harbach (2007) also summarised St John's (2007) equal weight and implied weight analyses, where *Culex* was non-monophyletic and relatively closely related to *Oculeomyia*. Lastly, Laurito and Almirón (2013) conducted a morphological analysis that once again supported the non-monophyly of *Culex* and the inclusion of *Oculeomyia*.

Within the morphological dataset of Harbach *et al*. (2012), many conserved characters were shared between the subgenera *Culex*, *Barraudius*, *Oculeomyia* and numerous other taxa. Nonetheless, some of the most exclusive shared characteristics included the absence of the fourth-instar larval 2a-S seta, the cephalad insertion of the pupal 9-VIII seta and the male's paraproct crown with a cluster of spicules. These three subgenera also overlap in portions of their geographic range. The subgenus *Culex* has a worldwide distribution and *Oculeomyia* occurs in the Afrotropical, Australian, Oriental and Palearctic Regions, where both partially overlap with *Barraudius'* Afrotropical and Palearctic occurrence (Mosquito Taxonomic Inventory 2021). The phylogenetic results may either support the inclusion of *Barraudius* and *Oculeomyia* within the subgenus *Culex* or justify the subdivision of the subgenus into several subgenera. However, both these perspectives may warrant further investigation and careful consideration for the potential implications associated with such taxonomic adjustments.

Most of the represented subgenus *Culex* species belonged to the Pipiens Group, which was consistently recovered as a non-monophyletic assemblage across all analyses. The phylogenetic results of several other authors also supported the non-monophyly of the Pipiens Group. Miller *et al*. (1996) investigated the phylogenetic relationships of the genus *Culex* based on ITS1 and ITS2 sequences. Here, the Sitiens Group was included within the larger Pipiens Group clade, where its sister relationship to a portion of the Pipiens Group was well-supported. Harbach's (2007) summary of St John's (2007) implied weight morphological analysis also recovered a non-monophyletic Pipiens Group. Within Harbach *et al*.'s (2017) own analyses, the Pipiens Group was spread over many polytomous clades within the collapsed tree. Here, individual members of the Pipiens Group was sister to the

subgenera *Allimanta* and *Barraudius*, and *Culex*'s Guiarti Group. The authors additionally noted that the morphological diversity and widespread distribution of the Pipiens Group likely support their non-monophyletic status. Lastly, the implied weight morphological analysis of Laurito and Almirón (2013) recovered a paraphyletic Pipiens Group with the inclusion of the Sitiens Group and *Oculeomyia*.

Several affiliations within the subgenus were supported by multiple analyses in the current study, including the grouping of the Trifilatus + Pipiens Subgroups and Apicinus Subgroup + Salinarius Complex. The relatively close relationship between the Trifilatus and Pipiens Subgroups was also recovered by several morphological analyses of Harbach *et al.* (2012), where the relationships were unsupported and non-monophyletic. This affiliation was also recovered in the combined ITS1 and ITS2 sequence data of Miller *et al.* (1996), which was once again unsupported. Most characters in the morphological dataset of Harbach *et al.* (2012) were shared between the Trifilatus and the Pipiens Subgroups and the widespread distribution of the Pipiens Subgroup likely overlaps with the Trifilatus Subgroup's Australasian, Palaearctic, Oriental and Afrotropical occurrence (Edwards 1932). Despite the shared characteristics and weak phylogenetic association between these taxa, additional investigations might clarify the relationship between these subgroups.

The affinity between the Apicinus Subgroup and Salinarius Complex was recognised by Edwards (1932) with the establishment of the Salinarius-Apicinus Series, although the author believed that this series represented an artificial grouping. This notion was reflected in the morphological analyses of Harbach *et al.* (2012), where one member of the Salinarius Complex was situated in close proximity to a species from the Apicinus Subgroup, while the remaining subgroup members were placed distantly. Additionally, a single species from the Salinarius Complex grouped with three members of the Apicinus Subgroup in the implied weight morphological results of Laurito and Almirón (2013), while the remaining members of both taxa were not affiliated with one another. Therefore, the close relationship between the Apicinus Subgroup and Salinarius Complex in the current results likely reflected the close affiliation between specific species, rather than the Apicinus Subgroup and Salinarius Complex as a whole.

The current results additionally recovered the Vishnui + Sitiens Subgroup clade in multiple analyses, which was well-supported in the COI results (Fig 3.14). Two additional studies also recovered a relatively close relationship between these subgroups. The NJ analyses of Li *et al.* (2020), based on COI nucleotide sequences, produced a clade consisting of the Sitiens Subgroup and the genus *Lutzia*, which was relatively closely related to the Vishnui Subgroup. Additionally, the morphological analysis of Laurito and Almirón (2013) produced a sister relationship between the Vishnui Subgroup and a clade consisting of the Sitiens Subgroup + *Oculeomyia*. However, the morphological analysis of Harbach *et al.* (2017) did not recover a close relationship between the Sitiens and Vishnui Subgroups, since little phylogenetic resolution was achieved within the collapsed tree. In this instance, their analysis produced a close relationship between species of the Sitiens, Gelidus and Mimeticus Subgroups. Therefore, this affiliation between the subgroups was inconsistent and generally non-monophyletic in other analyses.

Polyphyletic non-sampled South African *Culex* species (*Cx. neavei* & *Cx. simpsoni*)

Both *Cx.* (*Cux.*) *neavei* and *Cx.* (*Cux.*) *simpsoni* were represented by publicly available sequences in the COI analyses (Fig. 3.17 a), where both produced polyphyletic assemblages. Here, East African (Kenya & Malawi) members of both species grouped with *Cx.* (*Cux.*) *univittatus* (PP 95), while South African members grouped with *Cx.* (*Cux.*) *perexiguus* and *Cx.* (*Cux.*) *trifoliatus* (PP 100, BS 99). The aggregation of *Cx.* (*Cux.*) *perexiguus* sequences from three sets of authors provided some validity to their species identity, while the scattered *Cx.* (*Cux.*) *simpsoni* and *Cx.* (*Cux.*) *neavei* individuals may have been the product of misidentified specimens. However, only a single specimen of *Cx.* (*Cux.*) *trifoliatus* was included and the identity of this specimen could not be corroborated.

These patterns of phylogenetic groupings were also observed in their COI haplotypes, where the alleles were not associated with their assigned species. In each case, *Cx.* (*Cux.*) *neavei* showed a greater affinity towards *Cx.* (*Cux.*) *simpsoni* from the same geographic region than the geographically distant conspecific specimen. A few of the observed affiliations were also recovered in other publications, including the relatively close relationship between *Cx.* (*Cux.*) *univittatus*, *Cx.* (*Cux*) *perexiguus* and *Cx.* (*Cux.*) *simpsoni* in the morphological analyses of Harbach *et al.* (2012) (K = 16) and the well-supported and relatively close relationship between *Cx.* (*Cux.*) *univittatus* and *Cx.* (*Cux.*) *simpsoni* in the COI ML analysis of Ajamma *et al.* (2016). However, both Ajamma *et al.* (2016) and Harbach *et al.* (2017) did not recover *Cx.* (*Cux.*) *neavei'*s proximal placement to these species.

Nonetheless, both *Cx.* (*Cux.*) *neavei* and *Cx.* (*Cux.*) *simpsoni* share numerous morphological features (Jupp 1996) and parts of their range in southern and eastern Africa (Edwards 1932, Jupp 1971). However, since only two sequences from each species were included in the analyses, it was not possible to ascertain whether these divergent placements reflected taxonomic issues, misidentified specimens or the limitations of the selected gene regions.

Affiliations of sampled *Culex* species (*Cx. pipiens*, *Cx. quinquefasciatus*)

The two sampled members of the Pipiens Subgroup, *Cx.* (*Cux.*) *pipiens* and *Cx.* (*Cux.*) *quinquefasciatus*, were closely affiliated in all analyses and were often indistinguishable in the phylogenetic results. This was reflected in their haplotypes, where no alleles differentiated the two species, which may have reflected the limitations of the gene region or the inclusion of misidentified specimens. Additionally, these species formed non-monophyletic assemblages in COI analyses (Fig. 3.14). Here, sampled *Cx.* (*Cux.*) *pipiens* specimens formed a distinct lineage, which was sister to the well-supported remainder of the subgroup consisting of *Cx.* (*Cux.*) *quinquefasciatus* and non-sampled *Cx.* (*Cux.*) *pipiens* specimens. Despite the morphological overlap between these species, sequenced specimens of *Cx.* (*Cux.*) *pipiens* and *Cx.* (*Cux.*) *quinquefasciatus* were nonetheless morphologically and phylogenetically distinct from one another. Furthermore, the two species were clustered in conspecific groupings in the ITS2 results, with no apparent distinction between the sampled and non-

sampled *Cx.* (*Cux.*) *pipiens* specimens (Fig. 3.15). Finally, the 28S phylogenetic results produced no evident distinction between the two species (Fig. 3.16). In each case, the groupings of the South African specimens were not associated with their sampling locality or morphological variation.

When considering the haplotypes of presumed *Cx.* (*Cux.*) *pipiens* specimens, South African individuals were genetically distinct from non-South African specimens in the COI and ITS2 datasets (Table 3.12 & 3.13). The sampled specimens also displayed numerous unique SNPs in the COI region, while only a few polymorphic sites were observed in the ITS2 region. South African haplotypes were not noticeably associated with their sampling localities or morphology, which included the relatively dark and robust *Cx.* (*Cux.*) *pipiens* specimen (CLX12.10). However, the significant genetic distance between the Eurasian and South African specimens in the COI dataset reflected the geographic separation between these populations.

Conversely, the presumed *Cx.* (*Cux.*) *quinquefasciatus* specimens displayed minimal genetic variation in the COI region. Here, no alleles differentiated the various populations and SNPs often remained consistent across the various geographic regions. Nonetheless, the two morphologically atypical specimens (CLX4.2.19, CLX13.5) each possessed a unique haplotype, which was not evidently associated with their sampling localities or phylogenetic placements.

Many other studies supported the close relationship between *Cx.* (*Cux.*) *pipiens* and *Cx.* (*Cux.*) *quinquefasciatus*, while several analyses similarly recovered their polyphyletic structure. The bootstrap analyses (ML & NJ) of Shepard *et al.* (2006), based on the secondary structure alignment of 18S sequences, produced a close and well-supported relationship between *Cx.* (*Cux.*) *pipiens* and *Cx.* (*Cux.*) *quinquefasciatus*. The morphological analysis of Laurito and Almirón (2013) similarly recovered a close relationship between both species. Other studies included multiple specimens within their analyses, which often revealed their non-monophyletic structure. The ITS1 and ITS2 sequence analyses of Miller *et al.* (1996) produced polyphyletic *Cx.* (*Cux.*) *pipiens* and *Cx.* (*Cux.*) *quinquefasciatus* clades, with evidence of introgression between these species. This was similar to Ajamma *et al.*'s (2016) results based on COI and ITS2 sequences, where these species did not form monophyletic groupings. On the other hand, the UPGMA analysis of Weitzel *et al.* (2009), based on the allelic differentiation of multiple enzyme gene loci, sufficiently differentiated *Cx.* (*Cux.*) *pipiens* and *Cx.* (*Cux.*) *quinquefasciatus* specimens from multiple populations.

Since these species are morphologically alike, many authors have sought objective measures to distinguish between *Cx.* (*Cux.*) *pipiens* and *Cx.* (*Cux.*) *quinquefasciatus*, where the use of male genitalia has proven to be reliable (Jupp 1996). In an attempt to examine the presence of *Cx.* (*Cux.*) *pipiens* and *Cx.* (*Cux.*) *quinquefasciatus* hybrids in Argentina, Almirón *et al.* (1995) classified species according to the DV/D ratios of the male genitalia. The authors observed naturally occurring hybrids and successfully produced fertile hybrids through crossing experiments, which displayed intermediate DV/D ratios. Similar results were obtained by Barr and Kartman (1951) and Barr (1957), who also successfully

produced hybrids and observed specimens with intermediate characters in regions where the two species co-occur. Therefore, due to the morphological similarity and instances of gene flow between these taxa, numerous authors consider *Cx.* (*Cux.*) *pipiens* and *Cx.* (*Cux.*) *quinquefasciatus* as subspecies of *Cx.* (*Cux.*) *pipiens* (Subra 1981, Urbanelli *et al.* 1997, Diaz-Badillo *et al.* 2011).

Jupp (1978) investigated the specific status of these taxa in South Africa. The author cited differences in their morphology and mating behaviour, the infrequency of experimental cross-inseminations and the absence of naturally occurring hybrids as justifications for their specific status. Cornel *et al.* (2003) further confirmed the specific status of these species in South Africa, based on morphological and enzyme analyses, in conjunction with *Wolbachia* infection rates. This conclusion was once again supported by the phylogenetic separation of South African *Cx.* (*Cux.*) *pipiens* and *Cx.* (*Cux.*) *quinquefasciatus* specimens in the current COI results. However, it is worth noting that the specimens that were too morphologically ambiguous to classify were not sequenced in the present study, which may have represented genetically intermediate forms. Considering that these species were not monophyletic in the COI and 28S analyses, yet formed conspecific clusters in the ITS2 results, it is difficult to draw definitive conclusions regarding their specific status. Inferences were further impeded by the possible inclusion of misidentified specimens in these analyses. Nonetheless, the range of these species still overlaps in portions of the Afrotropical Region (Jupp 1996), and the potential for hybridisation remains. Such hybridisation may hold implications for the epidemiology of associated diseases, since these species have varying host affinities and pathogen associations (Cornel *et al.* 2003).

Affiliations of sampled *Culex* species (*Cx. theileri* & *Cx. univittatus*)

Multiple current analyses recovered the close and often well-supported affiliation between *Cx.* (*Cux.*) *theileri* and *Cx.* (*Cux.*) *univittatus*. However, due to the greater taxonomic coverage, the relationship between these species was interrupted by other favoured associations in the current COI results (Fig. 3.14). Likewise, this sister relationship between *Cx.* (*Cux.*) *theileri* and *Cx.* (*Cux.*) *univittatus* was not recovered by other authors. Based on the complete datasets, the morphological analyses of Harbach *et al.* (2012) recovered a close and well-supported relationship between *Cx.* (*Cux.*) *univittatus* and *Cx.* (*Cux*) *perexiguus*, which was in turn associated with *Cx.* (*Cux.*) *theileri*. However, the COI ML analysis of Ajamma *et al.* (2016) and the morphological analyses of Harbach *et al.* (2017) did not recover this affiliation between *Cx.* (*Cux.*) *theileri* and *Cx.* (*Cux.*) *univittatus*. Therefore, despite their proximal placement in the ITS2 and 28S results, these species are likely not closely related.

In the current analyses, *Cx.* (*Cux.*) *theileri* was consistently divided into separate South African and Eurasian lineages, while sampled specimens were not stratified by their sampling locality or instances of morphological variation. This was also reflected in the COI and ITS2 haplotypes (Table 3.15 & 3.16), where numerous alleles and indels distinguished the South African specimens from non-sampled specimens. Haplotypes were also not associated with their sampling locality within the Free State, while

three morphologically divergent specimens (CLX1.6, CLX2.21 & CLX2.24) possessed unique COI or ITS2 haplotypes, which nonetheless yielded no unique phylogenetic placements. Since sampled individuals formed well-supported groupings with conspecific individuals in all relevant analyses, it supported their species identities.

The COI results similarly produced a well-supported monophyletic *Cx.* (*Cux.*) *univittatus* clade (Fig. 3.14; PP 100; BS 96), where the non-South African specimen (Malawi) was situated relatively basally within the species clade. The species' monophyly thus supported the assigned identities of the sampled specimens. The South African specimens were not stratified by their sampling localities, while the morphology of one specimen affected its placement. This atypical specimen (CLX2.69) was situated distantly from other sampled specimens in the COI results, while it grouped firmly with other sampled specimens in the ITS2 results (Fig. 3.15). Here, the dark coloured specimen lacked sequence data for all polymorphic sites in the ITS2 dataset, which secured its placement with the other sampled specimens. The atypical specimen exhibited intermediary characteristics between *Cx.* (*Cux.*) *univittatus* and the closely related *Cx.* (*Cux.*) *neavei*. This female possessed a stripe on the midfemur, as seen in *Cx.* (*Cux.*) *univittatus*, while the hindfemur's stripe was relatively short, as expected in *Cx.* (*Cux.*) *neavei* (Fig. 3.6 q). Jupp (1971) noted that specimens with intermediate characteristic have previously been collected in the field, which may have been hybrids.

The COI haplotypes of *Cx.* (*Cux.*) *univittatus* consisted of numerous variable sites (Table 3.17). Several COI alleles were uniquely fixed for all South African specimens, while other alleles distinguished two separate South African lineages, regardless of their sampling locality. Since *Cx.* (*Cux.*) *univittatus* was one of the most abundant and widespread species in the current study, it may have been inadvertently transported to new regions. This could explain the presence of two distinct lineages, irrespective of their biogeography or morphology. The only morphological exception was the relatively dark specimen (CLX2.69), which possessed a markedly different COI haplotype, resulting in its distant placement in the COI results. Nonetheless, the COI BLAST query returned a 98.9% match with another South African *Cx.* (*Cux.*) *univittatus* specimen, thus supporting its species identity.

Paraphyly in non-sampled South African *Culiciomyia* species (*Cx. cinereus* & *Cx. nebulosus*)

The Nebulosus Group was represented by publicly available sequences in the COI dataset. The results recovered little phylogenetic distinction between *Cx.* (*Cui.*) *cinereus* and *Cx.* (*Cui.*) *nebulosus*, resulting in the paraphyly of *Cx.* (*Cui.*) *cinereus* in the ML results (Fig. 3.19). However, the group as a whole was stratified by the specimen geography, since all East African representatives of both species formed a single clade. Within the COI dataset, the group possessed minimal sequence variation, where no alleles differentiated the two species. Since these closely related species share portions of their range in the Afrotropical Region (Edwards 1932, Jupp 1996) and their morphological differences are either inconspicuous or specific to males and larvae (Jupp 1996), some specimens may have been

misidentified. Considering that no other relevant phylogenetic studies were available, additional investigations might clarify the relationship between *Cx.* (*Cui.*) *cinereus* and *Cx.* (*Cui.*) *nebulosus*.

Affiliations of sampled *Maillotia* species (*Cx. salisburiensis*)

Culex (*Mai.*) *salisburiensis* was represented by a single sampled specimen in the COI analyses, where its affiliations were either unresolved or unsupported (Fig. 3.14). Since only a single specimen was included in the dataset, conclusions regarding its phylogenetic placement were limited. However, this specimen was situated proximally to other members of the subgenus *Maillotia*, which provided a degree of support to its species identity. *Culex* (*Mai.*) *salisburiensis* shared a Bayesian polytomy with other members of *Maillotia*, *Eumelanomyia* and *Neoculex*, and was included in a weakly supported clade with *Cx.* (*Eum.*) *hayashii* + *Cx.* (*Eum.*) *rima* in the ML results. In light of these unsupported findings and the species' underrepresentation within phylogenetic studies, inferences on its affiliations were limited.

4.3.3 *ANOPHELES*

Subgenus *Anopheles*

The subgenus *Anopheles* was non-monophyletic in all current analyses, where the topology was inconsistent between the various DNA regions. Multiple authors have examined the phylogenetic relationships of the subgenus *Anopheles*, where it was frequently recovered as a non-monophyletic assemblage. The collapsed tree from the morphological analyses of Harbach and Kitching (2016) recovered a polyphyletic subgenus *Anopheles* that incorporated a clade of *Stethomyia* + *Bamaia* + *Bironella* and *Lophopodomyia*. However, these affiliations with the subgenus *Anopheles* were unsupported. The Bayesian COI sequence analysis of Wang *et al.* (2017) similarly recovered scattered subgenus *Anopheles* clades that were affiliated with several other subgenera. Both analyses of Harbach and Kitching (2005) also recovered a non-monophyletic subgenus *Anopheles* with a close relationship to *Lophopodomyia*. Furthermore, the equal weight analysis recovered affiliations with *Bamaia*, *Bironella* and *Stethomyia*, as defined by the current systematic framework

On the other hand, several other authors recovered a monophyletic subgenus *Anopheles*. This included Gholizadeh *et al.*'s (2013) analyses, based on ITS2 fragments, where it was represented by ten morphospecies. The molecular analysis of Foster *et al.* (2017), based on slow evolving mitochondrial protein sequences, also recovered a monophyletic subgenus *Anopheles* with a good degree of support. However, this analysis only included nine morphospecies. Lastly, the combined rDNA and mtDNA data analyses with ML and MP (maximum parsimony) of Sallum *et al.* (2002) recovered a monophyletic subgenus *Anopheles* with a significant degree of support, where it was also only represented by nine species. These monophyletic findings were consistently recovered with a relatively small number of representatives. Harbach and Kitching (2016) observed a similar trend and stated that the polyphyly of the subgenus *Anopheles* would likely be recovered with a sufficient taxonomic coverage.

The current study's results often produced a polyphyletic Laticorn Section intermixed with the Angusticorn Section. Several other studies similarly recovered Laticorn as a non-monophyletic assemblage. In the morphological analyses of Harbach and Kitching (2016), the Laticorn Section was associated with two species from Angusticorn's Cycloleppteron Series. This was similar to the equal weight analysis of Harbach and Kitching (2005), where a Cycloleppteron species was included within the paraphyletic Laticorn Section, while Laticorn was monophyletic in the implied weight analysis. The combined mtDNA and rDNA phylogenetic analyses of Sallum *et al.* (2002) also recovered three Laticorn species interspersed with Angusticorn clades. Finally, similar findings were observed in the successive weighting morphological analysis of Collucci and Sallum (2007) and the slow evolving mitochondrial protein sequence analysis of Foster *et al.* (2017), where Laticorn was paraphyletic with the inclusion of Angusticorn. In both the morphological datasets of Collucci and Sallum (2007) and Harbach and Kitching (2016), no characters could distinguish the Laticorn or Angusticorn Sections, while numerous features were shared between these taxa.

Multiple analyses in the current study also recovered a non-monophyletic Myzorhynchus Series, where its members often shared clades with the Anopheles Series' Punctipennis and Maculipennis Groups. The phylogenetic findings of other authors also supported these results. This included the results of Sallum *et al.* (2002), where numerous analyses based on ribosomal and mitochondrial DNA sequences recovered a close relationship between Myzorhynchus and the Punctipennis and Maculipennis Groups. A similar relationship was recovered in the studies of Harbach and Kitching, where their equal weight (Harbach & Kitching 2005) and parsimony (Harbach & Kitching 2016) analyses placed the Punctipennis Group basally to clades containing Myzorhynchus species. Many characters were shared between these taxa in the morphological dataset of Harbach and Kitching (2016), where one of the most exclusive features included the presence of several differentiated parabasal setae in males, arising from a prominent tubercle. These taxa also share portions of their distribution, where both the Punctipennis and Maculipennis Groups occur in the Nearctic and Neotropical Regions, while the Maculipennis Group and Myzorhynchus Series share a Palearctic distribution (Edwards 1932). Therefore, these findings may point to their shared evolutionary history.

Paraphyly in non-sampled South African *Anopheles* species (*An. coustani* & *An. tenebrosus*)

The Coustani Group was exclusively represented by publicly available sequences in the COI dataset. *Anopheles* (*Ano.*) *coustani* was paraphyletic with the inclusion of *An.* (*Ano.*) *tenebrosus*, where the topology was not associated with the specimen geography (Fig. 3.23). Here, the sequence data included conflicting characters, where some alleles differentiate the two species while others were shared between members of the two species. Similar phylogenetic findings were also recovered by Ciubotariu *et al.* (2020), where their COI ML analysis nested *An.* (*Ano.*) *tenebrosus* within the *An.* (*Ano.*) *coustani* clade.

Gillies and De Meillon (1968) noted that *An.* (*Ano.*) *tenebrosus* and *An.* (*Ano.*) *coustani* are morphologically distinct despite their sympatric occurrence, which serves as evidence for their genetic isolation. Despite the morphological variation of *An.* (*Ano.*) *tenebrosus*, the two species can still be distinguished by their wing and leg markings (Gillies & De Meillon 1968). Since other phylogenetic studies included single representatives of each species, it was not possible to verify whether these species belong to distinct clades. Therefore, additional investigations are needed to clarify the relationship between *An.* (*Ano.*) *coustani* and *An.* (*Ano.*) *tenebrosus*.

Subgenus *Cellia*

Cellia was represented by numerous species in the current analyses, where its structure, affiliations and monophyly was generally inconsistent. The species diversity and weak basal phylogenetic resolution likely contributed to its non-monophyletic structure within a few analyses. The findings of several other publications similarly produced conflicting results, where the majority of studies supported *Cellia*'s monophyly. This monophyly was well-supported in the morphological analysis of Harbach and Kitching (2016) and was also recovered in the morphological equal weight and implied weight analyses of Harbach and Kitching (2005). Sallum *et al.*'s (2002) combined rDNA data analyses and the mitochondrial protein sequence analysis of Foster *et al.* (2017) similarly produced monophyletic *Cellia* clades, but the analyses consisted of a small number of species.

On the other hand, at least two studies recovered the non-monophyly of *Cellia*. Within the ITS2 fragment analyses of Gholizadeh *et al.* (2013), the NJ tree produced a paraphyletic *Cellia* clade, while the taxon was monophyletic in the ML analysis. The Bayesian COI sequence analysis of Wang *et al.* (2017) similarly recovered scattered *Cellia* clades, which were weakly associated with several other subgenera. Therefore, the monophyly of *Cellia* seemed to be inconsistent or the product of weak taxon sampling. Harbach and Kitching (2016) nonetheless noted that the internal taxonomic structure of *Cellia* does not reflect its true evolutionary history, which was supported by the recovery of numerous non-monophyletic series in their results.

Multiple current analyses also produced polyphyletic and intermixed Funestus and Demeilloni Group clades, where the Marshallii and Wellcomei Groups were incorporated within the overarching Funestus Group clade in the ITS2 results (Fig. 3.21). Other authors similarly recovered various aspects of these affiliations. The morphological analysis of Harbach and Kitching (2016) recovered a polytomous clade within their collapsed tree that included the Demeilloni, Funestus, Marshallii and Wellcomei Groups, amongst several other taxa. The Funestus Group of Norris and Norris (2015) was similarly non-monophyletic, where both the COI and ITS2 analyses placed *An.* (*Cel.*) *theileri* (Wellcomei Group) within the Funestus clade. The Funestus Group was also polyphyletic in the Bayesian COI analysis of Wang *et al.* (2017), which may have been the product of the weak basal phylogenetic resolution. In the morphological dataset of Harbach and Kitching (2016), many characters were shared between the members of the Funestus, Demeilloni, Wellcomei and Marshallii Groups. The most exclusive of these

characters included the females' cibarial armature rods, the location of the premental apodeme and the structure of the spiracular apparatus in larvae. Within the Afrotropical Region, the Funestus Group is widespread and shares a large portion of its range with the Marshallii and Wellcomei Groups, while a smaller portion of this range overlaps with the Demeilloni Group (Gillies & De Meillon 1968). Therefore, considering the shared molecular and morphological features, it is likely that these taxa share a degree of common ancestry.

Two of the Funestus Group's subgroups (Funestus & Rivulorum) were also commonly associated with *An*. (*Cel*.) *sergentii* (Demeilloni Group) and *An*. (*Cel*.) *dthali* in the current results. These relationships were partly supported by the ITS2 fragment analyses of Gholizadeh *et al*. (2013), which produced a close and well-supported relationship between *An*. (*Cel*.) *sergentii* (Demeilloni Group) and *An*. (*Cel*) *dthali*. However, since few phylogenetic studies included the Funestus and Rivulorum Subgroups, their affiliations could not be substantiated.

Another frequently recovered Funestus Group clade consisted of the Minimus + Aconitus + Culicifacies Subgroups. This close relationship between the Minimus and Aconitus Subgroups was also recovered in the Bayesian COI sequence analysis of Wang *et al*. (2017), while multiple analyses supported their affiliation with the Culicifacies Subgroup. The NJ D3 sequence analysis of Swain *et al*. (2010) produced a close relationship between the Aconitus and Minimus subgroups, which was sister to a clade of *An*. (*Cel*.) *jeyporiensis* + Culicifacies Subgroup. The ML analysis of Yan *et al*. (2019), based on multiple protein-coding gene sequences, similarly recovered a close relationship between the three subgroups. However, in the studies of Swain *et al*. (2010) and Yan *et al*. (2019), these taxa were the only representatives of the Myzomyia Series and the affiliations between these subgroups were inevitable. Even so, the addition of the Rivulorum and Funestus Subgroups in the analyses of Garros *et al*. (2005) still produced a single clade consisting of the Minimus, Culicifacies and Aconitus Subgroups, where the latter two subgroups were closely related. These subgroups also share numerous morphological features, where the key of Rattanarithikul *et al*. (2006) listed their markings, upper proepisternal setae and the lack of specific scales as common features between the three subgroups. These subgroups share an Oriental distribution, while the Culicifacies and Minimus subgroups also occur in the Afrotropical Region (Edwards 1932, Coetzee 2020). Therefore, several findings support these taxa's shared affiliation.

Furthermore, the generally well-supported affiliations between *An*. (*Cel*.) *leesoni* and the Minimus and Fluviatilis Complexes were recovered in all relevant analyses. Several other studies recovered comparable results, including the NJ 28S (D3) sequence analysis of Swain *et al*. (2010), which produced a Minimus Subgroup clade consisting of the Minimus and Fluviatilis Complex species. Similar results were recovered by the D3 nucleotide sequence and NJ COII amino acid analyses of Garros *et al*. (2005), which produced a Minimus Complex + Fluviatilis Complex + *An*. (*Cel*.) *leesoni* clade. Finally, the COI and ITS2 sequence analyses of Norris and Norris (2015) recovered a close relationship between the Minimus Complex and *An*. (*Cel*.) *leesoni*. The affiliations between *An*. (*Cel*.) *leesoni* and

the Minimus and Fluviatilis Complexes were also supported in the morphological dataset of Garros *et al.* (2005), where these taxa shared three pupal features. This included the presence of three or more 2-Pa setae and the unique branching structure of seta 1-III and 5-III. Therefore, numerous findings support the grouping of *An.* (*Cel.*) *leesoni*, the Minimus Complex and the Fluviatilis Complex, which likely reflect a monophyletic subset of the Minimus Subgroup.

Subgenus *Cellia* - Pyretophorus Series

All current analyses recovered the Pyretophorus Series as a polyphyletic assemblage, where its affiliations were inconsistent between the various DNA regions. The structure of Pyretophorus was similarly inconsistent in the findings of several authors. The monophyly of this series was often associated with a low taxonomic coverage, as recovered in the COI and ITS2 sequence analyses of Norris and Norris (2015). The combined mtDNA and rDNA data phylogenetic analyses of Sallum *et al.* (2002) also only included four Pyretophorus species, where the combined mtDNA and one combined rDNA analyses produced a monophyletic series. However, the MP analysis of the combined rDNA data recovered Pyretophorus as a paraphyletic clade, with the inclusion of Myzomyia. The affiliations of Pyretophorus were unresolved in the collapsed cladogram of Harbach and Kitching (2016) since it shared a polytomy with several other *Cellia* series. The authors additionally expressed doubts regarding the monophyly of Pyretophorus. Therefore, additional comprehensive investigations are needed to confirm the structure of this series.

Polyphyletic non-sampled South African *Cellia* species (*An. longipalpis C* & *An. parensis*)

The Funestus Subgroup was monophyletic and well-supported in all relevant analyses. Conversely, two of its members (*An. longipalpis C* & *An. parensis*) were non-monophyletic in multiple analyses, with minimal phylogenetic distinction between the species in the ITS2 results (Fig. 3.28). The haplotypes yielded conflicting data, where a few COI alleles differentiated the two closely related species, while others were sporadically shared between their members. Conversely, no allele differentiated all members of *An.* (*Cel.*) *parensis* from *An.* (*Cel.*) *longipalpis C* in the ITS2 region.

Support for such non-monophyletic groupings was difficult to attain, since many studies only included single representatives of each species. These studies nonetheless supported the monophyly of the subgroup, including the Bayesian COI sequence results of Wang *et al.* (2017) and the 28S (D3) nucleotide and COII amino acid sequence results of Garros *et al.* (2005). The nucleotide sequence analyses of Norris and Norris (2015) also investigated the interspecific affiliations of the well-supported monophyletic Funestus Subgroup. The COI NJ results produced a weakly supported relationship between *An.* (*Cel.*) *funestus* and *An.* (*Cel.*) *longipalpis*, while the ITS2 MP and NJ results produced a well-supported clade consisting of *An.* (*Cel.*) *longipalpis* and *An.* (*Cel.*) *parensis*. These species were also included in the phylogenetic analyses of Koekemoer *et al.* (2009), based on ITS2 sequence data. The authors recovered two distinct ITS2 amplicons for *An.* (*Cel.*) *longipalpis C*, which consisted of a

larger and smaller fragment. These fragments had unique affiliations, where the short *An.* (*Cel.*) *longipalpis C* fragment was closely related to *An.* (*Cel.*) *parensis* with a high degree of support, while the other fragment was associated with *An.* (*Cel.*) *vaneedeni*. However, the third species, *An.* (*Cel.*) *funestus* was placed basally to both these groupings.

It is worth noting that the COI sequences generally tended to support the affiliation between *An.* (*Cel.*) *longipalpis C* and *An.* (*Cel.*) *funestus*, while the ITS2 sequences produced a close relationship between *An.* (*Cel.*) *longipalpis* and *An.* (*Cel.*) *parensis*. This discrepancy might have been caused by inherent differences in the phylogenetic signal between the two DNA regions. The study of Lv *et al.* (2014) investigated the intra- and interspecific divergences for the mitochondrial COI and the nuclear ITS2 DNA regions within another group of arthropods, ticks. The authors recovered a greater interspecific divergence within the ITS2 region, which contributes to this region's phylogenetic utility. However, the incorporation of additional gene regions might clarify the true affiliations of these mosquito species.

Paraphyly in non-sampled South African *Cellia* species (*An. gambiae* & *An. merus*)

The Gambiae Complex was represented by publicly available sequences from two different species. The complex was monophyletic and well-supported in all relevant analyses, while the phylogenetic distinction between its members was poor, resulting in the paraphyly of *An.* (*Cel.*) *gambiae* with the inclusion of *An.* (*Cel.*) *merus* in the COI results (Fig. 3.25). Several alleles were unique to each species within the COI and ITS2 datasets, while others were sporadically shared between its members.

Other authors have documented the limits of molecular data when investigating the phylogeny of the Gambiae Complex. Here, phylogenetic inferences are impeded by introgression, incomplete lineage sorting, incongruent results and the recent diversification of the Gambiae Complex (Besansky *et al.* 2003). Therefore, authors have instead focused on chromosomal inversions for phylogenetic inferences, which has recovered a close relationship between *An.* (*Cel.*) *gambiae* and *An.* (*Cel.*) *merus* (Besansky *et al.* 1994, Kamali *et al.* 2012). However, since the adult female members of the Gambiae Complex are morphologically inseparable (Coetzee 2020) and share parts of their range in the Afrotropical Region (Gillies & De Meillon 1968), it is possible that these database specimens may have been misidentified. Considering the limits of molecular and morphological data which affect the accuracy of phylogenetic results, no definitive conclusions can be made regarding the relationships of these species.

Paraphyly in non-sampled South African *Cellia* species (*An. cinereus*)

Anopheles (*Cel.*) *cinereus* was represented by two publicly available specimens in the ITS2 analysis, where they were paraphyletic with the inclusion of Paramyzomyia and a large portion of Pyretophorus (Fig. 3.27). A large degree of sequence variation was observed between the two conspecific specimens, which seemed disproportionate to the degree of geographic separation. This genetic difference likely contributed to the non-monophyletic structure of the species. However, since this species had a limited

representation within the current analyses, the phylogenetic structure of this species could not be confirmed.

Affiliations of sampled *Cellia* species (*An. cydippis* / *squamosus*)

All relevant analyses in the current study grouped the sampled *An.* (*Cel.*) *cydippis* / *squamosus* specimens with the publicly available *An.* (*Cel.*) *squamosus* sequences. This created well-supported monophyletic clades with distinct sampled and non-sampled lineages. However, the specific identity of the sampled specimens was uncertain, since no *An.* (*Cel.*) *cydippis* DNA sequences were available for comparison. *Anopheles* (*Cel.*) *cydippis* has not been documented in the Free State Province (Gillies & De Meillon 1968), yet the haplotypes of Free State specimens were markedly different from other African *An.* (*Cel.*) *squamosus* specimens (Table 3.18 & 3.19). This included several alleles that differentiated the populations in the COI region. Here, the two Bloemfontein specimens shared two other alleles, which reflected their phylogenetic placement (Fig. 3.24 a). However, their stratification was independent of morphology, since all sampled specimens were morphologically identical.

Additional affiliations of *An.* (*Cel.*) *squamosus* were inconsistent in the current study, since the species was only affiliated with *An.* (*Cel.*) *pharoensis* in the ITS2 results (Fig. 3.21). A few other publications likewise supported this affiliation, where the close and well-supported relationship between *An.* (*Cel.*) *squamosus* and *An.* (*Cel.*) *pharoensis* was recovered in the COI sequence analysis of Ajamma *et al.* (2016). This well-supported relationship was also recovered in the morphological analysis of Harbach and Kitching (2016). However, these affiliations were unsupported and inconsistent in the COI analyses of Norris and Norris (2015), where *An.* (*Cel.*) *squamosus* and *An.* (*Cel.*) *pharoensis* were recovered as sister taxa in the NJ analysis, yet associated with other taxa in the MP results. Due to the instability of this affiliation, no definitive conclusions could be made regarding the relationships of these taxa.

4.4 CONCLUDING REMARKS

Taxonomy has traditionally emphasised the use of morphological characteristics in the description and classification of species, which became an integral aspect of systematics. The morphological approach was vital in establishing the current culicid systematic framework, which has only recently been revolutionised with the advent of molecular technology. Molecular phylogeny has provided a powerful approach to investigate the otherwise cryptic affiliations between species. This method expanded the repertoire of phylogenetically informative characters, which illuminated some of the limitations of the morphological approach. Multiple culicid taxa are currently acknowledged as non-monophyletic assemblages and numerous phylogenetic efforts have attempted to elucidate their evolutionary history, to serve as a basis for their systematic classification.

Many prior comprehensive phylogenetic studies relied on morphological data (Reinert *et al*. 2009, Harbach *et al*. 2012, Harbach & Kitching 2016) and minor taxa were generally underrepresented within the phylogenetic literature. Molecular studies also often examined the generic and subfamilial relationships of taxa, rather than their intrageneric affiliations. Furthermore, studies focusing on South African species were also restricted to anophelines (Koekemoer *et al*. 2009, Norris & Norris 2015) and consisted of smaller datasets. Therefore, the current study aimed to examine the intrageneric affiliations of South African *Aedes*, *Culex* and *Anopheles* species within the broader systematic context by including representatives from a diverse array of taxonomic subdivisions.

The larger taxonomic divisions were often recovered as non-monophyletic within the current study, especially when a high taxonomic coverage was achieved. These results were also frequently supported by the findings from other morphological and molecular studies. This non-monophyly can be resolved by subdividing the constituent clades into separate taxa or incorporating the affiliated clades within the parent taxon. However, such taxonomic changes have downstream implications, especially regarding the retrieval of information relating to the amended taxa. To maintain the integrity and accessibility of relevant information, it is imperative to achieve a robust and well-supported taxonomic consensus before implementing such taxonomic changes.

Therefore, the main findings of this study are based on results recovered by multiple DNA regions and additionally supported by the findings of multiple other publications:

1. The results from the current study and those from multiple other authors recovered several polyphyletic *Neomelaniconion* species. This was evident in *Ae*. (*Neo*.) *mcintoshi*, which was commonly recovered as scattered clades associated with numerous other species. These non-monophyletic assemblages and the successful hybridisation of several *Neomelaniconion* species by Zavortink (1990) raised doubt about the specific status of these species. A comprehensive molecular and morphological investigation into *Neomelaniconion* and its intraspecific variation would likely clarify the boundaries between these species.

2. All current analyses recovered *Aedimorphus* as a polyphyletic assemblage, in addition to its weak affiliation with *Stegomyia*. However, this non-monophyly and residual affiliation were inconsistently supported by the results of other authors. These subgenera may still share a relatively deep and obscured phylogenetic history, which could be clarified with the use of relatively conserved genes.

3. The recovered monophyly of *Stegomyia* was inconsistent within the literature, where both the current results and several other authors recovered the subgenus as a polyphyletic assemblage. Despite this disparity, further investigations may be necessary to confirm its monophyletic status or support its division into monophyletic subunits. Nonetheless, numerous sources supported the relatively close relationship between its Simpsoni and Metallicus Groups.

4. *Ochlerotatus* was consistently recovered as a polyphyletic assemblage within the current results, which was similarly supported by the findings of other authors. Reinert *et al.* (2008) aimed to establish a monophyletic *Ochlerotatus* by recognising the associated clades as divisions of *Ochlerotatus*. Despite their efforts, the non-monophyly of the subgenus appears to persist.

5. The non-monophyly of the subgenus *Culex* was ubiquitous across all DNA regions and multiple analyses supported the inclusion of *Barraudius* and *Oculeomyia* within the overarching clade. This inclusion was also recovered by numerous other authors and may either necessitate the subdivision of the subgenus *Culex* or the inclusion of these taxa within the subgenus. The polyphyly of the Pipiens Group also coincided with the polyphyly of *Culex* in the current results, and was recovered in the findings of other authors. Therefore, these results exemplify the need to re-examine the subgenus *Culex*'s internal systematic structure.

6. The inclusion of multiple *Cx*. (*Cux*.) *pipiens* and *Cx*. (*Cux*.) *quinquefasciatus* specimens often yielded polyphyletic species groupings, which was observed in the findings of other authors and the current COI and 28S results. The evidence of non-monophyly in conjunction with naturally occurring hybrids once again raises doubts regarding the specific status of these species. Although sampled specimens formed distinct clusters within the current analyses, this was likely due to the exclusion of morphologically ambiguous specimens. The molecular investigation of such intermediary specimens would likely clarify the specific status of these species within South Africa.

7. The subgenus *Anopheles* was non-monophyletic in all current analyses, which contributed to the polyphyly of its Laticorn Section. The non-monophyly of both taxa was also recovered by numerous other authors. Since the current structure of the subgenus *Anopheles* does not seem to reflect the evolutionary history of its taxa, the subgenus may benefit from a comprehensive re-evaluation.

8. Numerous aspects of the subgenus *Cellia*'s structure were recovered within current results and supported by the findings of other authors. This included the polyphyly of the Funestus Group, where it was associated with numerous other taxa in the current analyses. This group likely does not reflect the natural affiliations of its members, and focused investigations may reveal the constituent monophyletic subdivisions of the Funestus Group.

In addition to the listed phylogenetic findings, this study also provided insight into the affiliations of numerous South African species. To the best of the author's knowledge, this study provided the first molecular phylogenetic investigations of *Ae*. (*Neo.*) *luridus*, *Ae*. (*Neo.*) *luteolateralis*, *Ae*. (*Stg.*) *contiguus*, *Ae*. (*Och.*) *caballus*, *Ae*. (*Cat.*) *mixtus* and *Cx*. (*Mai.*) *salisburiensis*. Furthermore, the current study also generated distribution data for all the sampled species, where several species were documented in the Free State Province for the first time. The presence of additional species in the region may have implications for the epidemiology of any associated diseases. Additionally, since the distribution of vector species may shift due to climate change, the generated distribution data can serve as a basis for future investigations regarding range expansions and vector control efforts.

In conclusion, the current study highlights and supports consistent findings within the phylogenetic literature, revealing numerous challenges within the current systematic framework. These taxonomic challenges may be resolved in future studies with the joint incorporation of molecular and morphological data, and reliance on comprehensive taxonomic datasets. Such comprehensive studies would likely depend on the availability of molecular data across several gene regions, which is currently limited within molecular databases. However, as the repertoire of sequences steadily increases over time, the application of multigene phylogenetic investigations may become a viable option in numerous taxa. The current study expanded the available molecular data for multiple South African species (MPSAM001-21 - MPSAM062-21) and provided updated distribution data for all of the sampled taxa. The generated results expanded our knowledge on South African mosquitoes, contributing to the foundation of phylogenetic and ecological data that can be incorporated into other epidemiological, biogeographical and evolutionary investigations.

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5. APPENDICES

5.1 APPENDIX 1

Environmental & Biosafety Research Ethics Committee

15-Aug-2018

Dear **Miss Liezl Whitehead**

Project Title: **Phylogenetic analysis of South African Aedes, Anopheles and Culex (Diptera: Culicidae) based on COI, ITS2 and ND4 sequences**

Department: **Zoology and Entomology (Bloemfontein Campus)**

APPLICATION APPROVED

This letter confirms that this research proposal was given ethical clearance by the Biosafety & Environmental Research Ethics Committee of the University of the Free State.

Your ethical clearance number, to be used in all correspondence is: **UFS-ESD2018/0004**

Please note the following:

1. **This ethical clearance is valid for one year from the issuance of this letter.**
2. **If the research takes longer than one year to complete, please submit a Continuation Report to the Ethics Committee before ethical clearance expires.**
3. **If any changes are made during the research process (including a change in investigators), please inform the Ethics Committee by submitting an Amendment.**
4. **When the research is concluded, please submit a Final Report to the Ethics Committee.**

Thank you for your application and we wish you well in all of your research endeavours.

Yours Sincerely

Prof. RR (Robert) Bragg
Chairperson: Biosafety & Environmental Research Ethics Committee
University of the Free State

Directorate: Research Development
T: +27 (0)51 401 9398 | +27 (0)51 401 2075 | E: smitham@ufs.ac.za
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5.2 APPENDIX 2

OFFICIAL PERMIT

113 ST ANDREW STREET
ST ANDREW BUILDING
GROUND FLOOR
BLOEMFONTEIN, 9300
FREE STATE
SOUTH AFRICA

PERMIT OFFICE
PRIVATE BAG X20801
BLOEMFONTEIN, 9300

TEL: +27 (0) 51 400 9527/26
TEL: +27 (0) 51 400 9536

destea
department of economic, small business development, tourism and environmental affairs
FREE STATE PROVINCE

PERMIT HOLDER DETAILS

ID NUMBER	
NAME	Liezl Whitehead
PHYSICAL ADDRESS	
POSTAL ADDRESS	
MOBILE NUMBER:	
TEL NUMBER	
EMAIL ADDRESS	

THIS PERMIT IS ISSUED IN TERMS OF THE NATIONAL ENVIRONMENTAL MANAGEMENT: BIODIVERSITY ACT (10 OF 2004) (THREATENED OR PROTECTED SPECIES REGULATIONS) AND IN TERMS OF THE FREE STATE NATURE CONSERVATION ORDINANCE (8 OF 1969).
AUTHORISATION IS HEREBY GRANTED TO THE HOLDER OF THIS PERMIT TO;

Collect the following species at Gariep Nature Reserve and Willem Pretorius Nature Reserve for research purpose.

Species	Scientific name	Total
Insects, arachnids, myriapods & crustaceans	Arthropoda	Will depend on sampling

PERMIT HOLDER'S SIGNATURE	APPROVED BY THE MEC DEPARTMENT OF ECONOMIC, SMALL BUSINESS DEVELOPMENT, TOURISM AND ENVIRONMENTAL AFFAIRS	
EXPIRY DATE 31 December 2020 RETURN PERMIT AFTER EXPIRY	PERMIT NUMBER JM 5040/2018	DATE ISSUED 11 October 2018

Department of Economic & Small Business Development Tourism & Environmental Affairs
06 NOV 2018
Private bag X 20801
Bloemfontein 9300
Free State Province

OFFICIAL PERMIT

113 ST ANDREW STREET ST ANDREW BUILDING GROUND FLOOR BLOEMFONTEIN, 9300 FREE STATE SOUTH AFRICA	PERMIT OFFICE PRIVATE BAG X20801 BLOEMFONTEIN, 9300 TEL: +27 (0) 51 400 9527/26 TEL: +27 (0) 51 400 9536	destea department of economic, small business development, tourism and environmental affairs FREE STATE PROVINCE

PERMIT HOLDER DETAILS

ID NUMBER			
NAME	Liezl Whitehead		
PHYSICAL ADDRESS		POSTAL ADDRESS	
MOBILE NUMBER:			
TEL NUMBER			
EMAIL ADDRESS			

THIS PERMIT IS ISSUED IN TERMS OF THE NATIONAL ENVIRONMENTAL MANAGEMENT: BIODIVERSITY ACT (10 OF 2004) (THREATENED OR PROTECTED SPECIES REGULATIONS) AND IN TERMS OF THE FREE STATE NATURE CONSERVATION ORDINANCE (8 OF 1969). AUTHORISATION IS HEREBY GRANTED TO THE HOLDER OF THIS PERMIT TO;

Collect the following species at Soetdoring nature reserve, Sterkfontein dam nature reserve, Kalkfontein dam nature reserve, Rustfontein nature reserve, Seekoevlei nature reserve and Tussen-die-Riviere nature reserve for research purpose.

Species	Scientific name	Total
Insects, arachnids, myriapods & Crustaceans	Arthropoda	Will depend on sampling

The permit holder is authorised to collect insects, subject to the conditions at the back.

PERMIT HOLDER'S SIGNATURE	APPROVED BY THE MEC DEPARTMENT OF ECONOMIC, SMALL BUSINESS DEVELOPMENT, TOURISM AND ENVIRONMENTAL AFFAIRS	
EXPIRY DATE 31 December 2020 RETURN PERMIT AFTER EXPIRY	PERMIT NUMBER JM 5040/2019	DATE ISSUED 04 February 2019

Department of Economic & Small Business Development Tour... & Environmental Affairs

08 FEB 2019

Private bag X 20801
Bloemfontein 9300
Free State Province

5.3 APPENDIX 3

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/4

Ms Liezl Whitehead
Room 212
Biology Building
University of the Free State

Dear Ms Whitehead,

RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Your fax / memo / letter/ Email dated 07 November 2018, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
3. As the specific collection areas within the Free State Province were not identified within the application, written permission must be obtained from the responsible state veterinarian or the Director Veterinary Services Free State Province prior to the collection of mosquitoes, in order to ensure that the area of collection is not under restriction for disease control purposes;
4. Mosquitoes may not be collected from provinces other than the Free State Province. Should the need arise to expand the collection area beyond the borders of the Free State Province, an application must be submitted for an amendment to this approval providing the relevant state veterinary letters;
5. Only dead mosquitoes may be transported from the collection site to the University of the Free State;
6. The DNA extraction must be performed within a Class II biosafety cabinet of which the calibration is up to date;

7. Compass Medical Waste Services must be used as accredited waste management company;
8. Isolated DNA may be stored at the University of the Free State;
9. Voucher specimens may only be deposited within the National Museum in Bloemfontein if point 1 above has been complied with for each collection performed within the Free State Province.

Title of research/study: Molecular phylogeny of South African *Aedes, Anopheles* and *Culex* (Diptera: Culicidae) based on *COI, ITS2* and *28S* DNA sequences
Researcher (s): Ms Liezl Whitehead
Institution: University of the Free State
Your Ref./ Project Number: UFS-ESD2018/0004
Our ref Number: 12/11/1/4

Kind regards,

MMaja.

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2019 -01- 28

5.4 APPENDIX 4

SPECIES	TAXON AUTHOR(S)	DNA REGION	ACCESSION / SPECIMEN NUMBER	SEQUENCE SOURCE	LOCALITY
			Aedes		
			Ingroup		
Ae. aegypti	(Linnaeus, 1762)	COI	AED14.1.1; MPSAM036-21	Sampled	Bothaville
		COI	AED18.11; MPSAM040-21	Sampled	Bloemfontein Urban Habitat 1
		COI	AY056596	GenBank	Laboratory Colony
		COI	HQ991718	GenBank	Chile
		COI	HQ991722	GenBank	Chile
		ITS2	JX423805	GenBank	Saudi Arabia
		ITS2	KF471585	GenBank	USA
		ITS2	AY512667	GenBank	Peru
		28S	DQ397937	GenBank	Indonesia
		28S	AY433205	GenBank	Laboratory Colony
Ae. albocephalus	(Theobald, 1903)	ITS2	JX282415	GenBank	Seychelles
		ITS2	JX282416	GenBank	Seychelles
		ITS2	JX282417	GenBank	Seychelles
Ae. albopictus	(Skuse, 1895)	COI	GQ143719	GenBank	Australia
		COI	HM102286	GenBank	USA
		COI	EU259306	GenBank	India
		ITS2	AF305554	GenBank	China
		ITS2	M95127	GenBank	USA
		ITS2	AY741376	GenBank	Greece
		28S	MT808421	GenBank	Thrace
		28S	DQ397934	GenBank	Japan
		28S	L22060	GenBank	
Ae. alternans	(Westwood, 1835)	COI	KU495045	GenBank	Australia
		COI	MG712511	GenBank	Australia
		COI	MG712513	GenBank	Australia
		ITS2	KU495671	GenBank	Australia
		ITS2	KU495675	GenBank	Australia
		ITS2	KX866179	GenBank	Australia
Ae. argenteopunctatus	(Theobald, 1901)	COI	MG242468	GenBank	Senegal
		COI	LC473670	GenBank	Malawi
		COI	MN552301	GenBank	Guinea
Ae. atropalpus	(Coquillett, 1902)	COI	GU907844	GenBank	Canada
		COI	JF868951	GenBank	Canada
		COI	MG242470	GenBank	USA
		ITS2	MG232605	GenBank	USA
		ITS2	KF471638	GenBank	USA
		ITS2	KF471637	GenBank	USA
Ae. australis	(Erichson, 1842)	COI	MONSW057-17	BOLD Systems	Australia
		COI	MG385731	GenBank	New Zealand
		COI	MONSW058-17	BOLD Systems	Australia
		ITS2	AF044507	GenBank	Australia
		ITS2	AF044518	GenBank	Australia
Ae. bahamensis	Berlin, 1969	COI	JX259530	GenBank	Bahamas
		COI	JX259531	GenBank	Bahamas
		ITS2	JN020552	GenBank	
		ITS2	JN020553	GenBank	
Ae. bancroftianus	Edwards, 1921	COI	KU495046	GenBank	Australia
		COI	MG242472	GenBank	Australia
		COI	KU495048	GenBank	Australia
		ITS2	KU495676	GenBank	Australia
		ITS2	KX866189	GenBank	Australia
		ITS2	KX866181	GenBank	Australia
Ae. bromeliae	(Theobald, 1911)	COI	MG242474	GenBank	Kenya
		COI	KT998410	GenBank	Uganda
		COI	KT998415	GenBank	Uganda
		ITS2	KT998430	GenBank	Uganda
		ITS2	MG232609	GenBank	Kenya
		ITS2	KF135510	GenBank	Mayotte

Ae. caballus	(Theobald, 1912)	COI	AED18.4; MPSAM038-21	Sampled	Bloemfontein Urban Habitat 1
		COI	AED18.14; MPSAM042-21	Sampled	Bloemfontein Urban Habitat 1
		COI	AED19.3; MPSAM045-21	Sampled	Bloemfontein Urban Habitat 2
		COI	AED19.11; MPSAM046-21	Sampled	Bloemfontein Urban Habitat 2
		COI	AED20.2707; MPSAM052-21	Sampled	Bloemfontein Semi-Pristine Habitat 2
		COI	AED21.1; MPSAM054-21	Sampled	Bloemfontein Urban Habitat 3
		COI	MH634431	GenBank	Iran
		COI	MH634434	GenBank	Iran
		COI	MH709107	GenBank	Iran
		ITS2	AED18.4; MPSAM038-21	Sampled	Bloemfontein Urban Habitat 1
		ITS2	AED19.11; MPSAM046-21	Sampled	Bloemfontein Urban Habitat 2
		ITS2	AED19.3; MPSAM045-21	Sampled	Bloemfontein Urban Habitat 2
		ITS2	AED20.2707; MPSAM052-21	Sampled	Bloemfontein Semi-Pristine Habitat 2
		ITS2	AED21.1; MPSAM054-21	Sampled	Bloemfontein Urban Habitat 3
		ITS2	MN158185	GenBank	Iran
		ITS2	MN158188	GenBank	Iran
		ITS2	MN158189	GenBank	Iran
Ae. cinereus	Meigen, 1818	COI	JX259550	GenBank	USA
		COI	KF761595	GenBank	Canada
		COI	JF876572	GenBank	Canada
		ITS2	AM397836	GenBank	
		ITS2	AM397837	GenBank	
		ITS2	MG232614	GenBank	USA
Ae. circumluteolus	(Theobald, 1908)	COI	KU187017	GenBank	Kenya
		COI	KU187016	GenBank	Kenya
		ITS2	FM211132	GenBank	Madagascar
		ITS2	FM211133	GenBank	South Africa
		ITS2	FM211134	GenBank	Madagascar
Ae. contiguus	Edwards, 1936	COI	AED9.2; MPSAM027-21	Sampled	Bethulie
Ae. cumminsii	(Theobald, 1903)	COI	KU187000	GenBank	Kenya
		COI	MG242484	GenBank	Senegal
		COI	MK300225	GenBank	Kenya
Ae. dentatus	(Theobald, 1904)	ITS2	AED12.2; MPSAM031-21	Sampled	Memel
		28S	AED12.2; MPSAM031-21	Sampled	Memel
Ae. detritus	(Haliday, 1833)	COI	KM258319	GenBank	Belgium
		COI	KC602685	GenBank	United Kingdom
		COI	KT876465	GenBank	Romania
		ITS2	MG232616	GenBank	Greece
		ITS2	KJ661028	GenBank	France
		ITS2	MN947506	GenBank	Tunisia
		28S	MT808422	GenBank	Thrace
Ae. diantaeus	Howard, Dyar & Knab, 1913	COI	JF876480	GenBank	Canada
		COI	JX259583	GenBank	USA
		COI	JX040504	GenBank	Sweden
		ITS2	MG232617	GenBank	Canada
		ITS2	KF535029	GenBank	Canada
		ITS2	KF535030	GenBank	Canada
Ae. eritreae	Lewis, 1942	COI	AED22.16; MPSAM057-21	Sampled	Bloemfontein Semi-Pristine Habitat 3
Ae. fowleri	(de Charmoy, 1908)	COI	GBDP50652-19	BOLD Systems	Senegal
		COI	MG242493	GenBank	Senegal
		COI	MN552299	GenBank	Guinea

Ae. fryeri	(Theobald, 1912)	28S	AED18.12; MPSAM041-21	Sampled	Bloemfontein Urban Habitat 1
Ae. furcifer	(Edwards, 1913)	COI	KU187186	GenBank	Kenya
		COI	MG242495	GenBank	Senegal
Ae. hirsutus	Brunhes & Boussès, 2017	COI	KU186993	GenBank	Kenya
		COI	MG242499	GenBank	Senegal
		COI	LC507875	GenBank	Ghana
Ae. japonicus	(Theobald, 1901)	COI	HQ978777	GenBank	USA
		COI	FJ641869	GenBank	Belgium
		COI	GU907918	GenBank	Canada
		ITS2	FJ403047	GenBank	
		ITS2	KF471613	GenBank	Switzerland
		ITS2	GU121103	GenBank	USA
		28S	DQ397931	GenBank	Japan
Ae. juppi	McIntosh, 1973	COI	AED2.1; MPSAM004-21	Sampled	Bloemfontein Smallholding 2
		COI	AED9.1; MPSAM026-21	Sampled	Bethulie
		COI	AED12.1; MPSAM030-21	Sampled	Memel
		COI	AED18.9; MPSAM039-21	Sampled	Bloemfontein Urban Habitat 1
		COI	AED20.107; MPSAM048-21	Sampled	Bloemfontein Semi-Pristine Habitat 2
		COI	AED20.2704; MPSAM051-21	Sampled	Bloemfontein Semi-Pristine Habitat 2
		ITS2	AED18.9; MPSAM039-21	Sampled	Bloemfontein Urban Habitat 1
		ITS2	AED20.2704; MPSAM051-21	Sampled	Bloemfontein Semi-Pristine Habitat 2
		28S	AED2.1; MPSAM004-21	Sampled	Bloemfontein Smallholding 2
		28S	AED18.9; MPSAM039-21	Sampled	Bloemfontein Urban Habitat 1
Ae. leesoni	Edwards, 1932	COI	CULSA040-19	BOLD Systems	South Africa
		COI	CULSA055-19	BOLD Systems	South Africa
Ae. luridus	McIntosh, 1971	COI	AED20.1720; MPSAM050-21	Sampled	Bloemfontein Semi-Pristine Habitat 2
		ITS2	AED20.1720; MPSAM050-21	Sampled	Bloemfontein Semi-Pristine Habitat 2
Ae. luteolateralis	(Theobald, 1901)	COI	AED22.28; MPSAM060-21	Sampled	Bloemfontein Semi-Pristine Habitat 3
Ae. mcintoshi	Huang, 1985	COI	AED20.2710; MPSAM053-21	Sampled	Bloemfontein Semi-Pristine Habitat 2
		COI	AED22.19; MPSAM059-21	Sampled	Bloemfontein Semi-Pristine Habitat 3
		COI	KJ940679	GenBank	Kenya
		COI	LC473696	GenBank	Malawi
		COI	KJ940551	GenBank	Kenya
		ITS2	AED20.2710; MPSAM053-21	Sampled	Bloemfontein Semi-Pristine Habitat 2
		ITS2	KJ940769	GenBank	Kenya
		ITS2	KJ940770	GenBank	Kenya
		ITS2	KJ940771	GenBank	Kenya
Ae. cf. *mcintoshi*	Huang, 1985	COI	AED20.405; MPSAM049-21	Sampled	Bloemfontein Semi-Pristine Habitat 2
		ITS2	AED20.405; MPSAM049-21	Sampled	Bloemfontein Semi-Pristine Habitat 2
Ae. metallicus	(Edwards, 1912)	COI	KU187014	GenBank	Kenya
		COI	KU380347	GenBank	Kenya
		COI	MG242506	GenBank	Senegal
		ITS2	KU056497	GenBank	Kenya
		ITS2	MG232628	GenBank	Senegal
		ITS2	KU056495	GenBank	Kenya
Ae. mixtus	Edwards, 1936	COI	AED4.2.2; MPSAM016-21	Sampled	Willem Pretorius Game Reserve

Ae. nipponicus	La Casse & Yamaguti, 1948	COI	KT358404	GenBank	South Korea
		COI	LC054374	GenBank	Japan
		COI	LC054375	GenBank	Japan
		ITS2	FJ534552	GenBank	
		28S	DQ397932	GenBank	Japan
Ae. notoscriptus	(Skuse, 1889)	COI	JF872402	GenBank	Australia
		COI	HQ945414	GenBank	Australia
		COI	MG242508	GenBank	Australia
		ITS2	KU495701	GenBank	Australia
		ITS2	KX866234	GenBank	Australia
		ITS2	MG232629	GenBank	
Ae. ochraceus	(Theobald, 1901)	COI	LC473674	GenBank	Malawi
		COI	KJ940693	GenBank	Kenya
		COI	LC473673	GenBank	Malawi
		ITS2	KJ940828	GenBank	Senegal
		ITS2	KJ940829	GenBank	Senegal
		ITS2	KJ940830	GenBank	Senegal
Ae. palpalis	(Newstead, 1907)	ITS2	FM211130	GenBank	Central African Republic
Ae. pertinax	Grabham, 1906	ITS2	MG232630	GenBank	USA
		ITS2	KP262404	GenBank	Jamaica
		ITS2	KP262407	GenBank	USA
Ae. phoeniciae	Coluzzi & Sabatini, 1968	ITS2	KX009741	GenBank	Cyprus / Turkey
Ae. provocans	(Walker, 1848)	COI	GU907923	GenBank	Canada
		COI	JF873432	GenBank	Canada
		COI	JN301652	GenBank	Canada
		ITS2	MG232631	GenBank	USA
		ITS2	KF535067	GenBank	Canada
		ITS2	KF535066	GenBank	Canada
Ae. quasiunivittatus	(Theobald, 1901)	COI	LC473675	GenBank	Malawi
Ae. rubrithorax	(Macquart, 1850)	COI	MG712527	GenBank	Australia
		COI	MONSW017-17	BOLD Systems	Australia
		COI	KU495029	GenBank	Australia
		ITS2	KX866092	GenBank	Australia
		ITS2	KU495664	GenBank	Australia
		ITS2	KX866090	GenBank	Australia
Ae. scapularis	(Rondani, 1848)	COI	MF172265	GenBank	French Guiana
		COI	MN997484	GenBank	Colombia
		COI	KT766398	GenBank	Colombia
		ITS2	FJ156758	GenBank	Brazil
		ITS2	FJ439563	GenBank	Brazil
		ITS2	FLMO015-20	BOLD Systems	USA
Ae. sierrensis	(Ludlow, 1905)	COI	JF868912	GenBank	Canada
		COI	MG242513	GenBank	USA
		COI	JX259670	GenBank	USA
		ITS2	MG232634	GenBank	USA
Ae. simpsoni	(Theobald, 1905)	COI	KT881399	GenBank	Kenya
		COI	LC473669	GenBank	Malawi
		ITS2	M95130	GenBank	Uganda
		ITS2	AF439789	GenBank	South Africa
		ITS2	AF158187	GenBank	Uganda
Ae. sudanensis	(Theobald, 1908)	COI	CULSA051-19	BOLD Systems	South Africa
		COI	MG242517	GenBank	Senegal
Ae. taeniorhynchus	(Wiedemann, 1821)	COI	JX259676	GenBank	Mexico
		COI	MG242518	GenBank	USA
		COI	KT766538	GenBank	Colombia
		ITS2	MG232636	GenBank	USA
Ae. tarsalis	(Newstead, 1907)	COI	MK533636	GenBank	Kenya
		COI	MK510879	GenBank	Kenya
		COI	KU187012	GenBank	Kenya
Ae. togoi	(Theobald, 1907)	COI	KC510142	GenBank	
		COI	KM497421	GenBank	China
		COI	AB738159	GenBank	
		ITS2	MW046044	GenBank	South Korea
		ITS2	EU980394	GenBank	
		28S	LC025777	GenBank	Japan

		28S	LC025768	GenBank	Japan
		28S	LC025729	GenBank	Japan
Ae. tremulus	(Theobald, 1903)	COI	KU495036	GenBank	Australia
		COI	KU495037	GenBank	Australia
		COI	KU495034	GenBank	Australia
		ITS2	KU495666	GenBank	Australia
		ITS2	KX866170	GenBank	Australia
		ITS2	KX866171	GenBank	Australia
Ae. triseriatus	(Say, 1823)	COI	MG242523	GenBank	USA
		COI	JX259685	GenBank	USA
		COI	GU907969	GenBank	Canada
		ITS2	AF166254	GenBank	USA
		ITS2	MG232639	GenBank	USA
		ITS2	MF002494	GenBank	USA
Ae. unidentatus	McIntosh, 1971	COI	AED8.30; MPSAM024-21	Sampled	Bethlehem
		COI	AED22.18; MPSAM058-21	Sampled	Bloemfontein Semi-Pristine Habitat 3
Ae. unilineatus	(Theobald, 1906)	COI	KU351084	GenBank	Iran
		COI	KJ768162	GenBank	Pakistan
		COI	KF406626	GenBank	Pakistan
Ae. vexans	(Meigen, 1830)	COI	HQ944964	GenBank	USA
		COI	HQ978776	GenBank	USA
		COI	GU907989	GenBank	Canada
		ITS2	AF298626	GenBank	China
		ITS2	EF539857	GenBank	Iran
		ITS2	M95132	GenBank	Laboratory Colony
		28S	MT808428	GenBank	Thrace
Ae. vittatus	(Bigot, 1861)	COI	MG242527	GenBank	Senegal
		COI	MF429950	GenBank	Spain
		COI	MH427551	GenBank	Laos
			Outgroup		
Ar. obturbans	(Walker, 1859)	ITS2	AF305557	GenBank	China
Ar. subalbatus	(Coquillett, 1898)	COI	HQ398903	GenBank	Thailand
		COI	AY729986	GenBank	India
		ITS2	KU497620	GenBank	
		28S	AY440287	GenBank	
		28S	AY440281	GenBank	
			Culex		
			Ingroup		
Cx. adamesi	Sirivanakarn & Galindo, 1980	COI	FGMOS2759-20	BOLD Systems	French Guiana
		COI	FGMOS2765-20	BOLD Systems	French Guiana
		ITS2	AY633719	GenBank	Peru
		ITS2	AY633720	GenBank	Peru
Cx. aliciae	Duret, 1953	COI	KX779780	GenBank	Brazil
		COI	KX779782	GenBank	Brazil
		COI	KX779781	GenBank	Brazil
		ITS2	GU299683	GenBank	Brazil
		ITS2	GU299684	GenBank	Brazil
Cx. annulioris	Theobald, 1901	COI	GQ165805	GenBank	Uganda
		COI	KU187019	GenBank	Kenya
		COI	MK533640	GenBank	Kenya
Cx. annulirostris	Skuse, 1889	COI	HQ561025	GenBank	Australia
		COI	HM909634	GenBank	Australia
		COI	JF872519	GenBank	Australia
		ITS2	KU495631	GenBank	Australia
		ITS2	KX865982	GenBank	Australia
		ITS2	KX865976	GenBank	Australia
Cx. antennatus	(Becker, 1903)	COI	LC473659	GenBank	Malawi
		COI	LC473660	GenBank	Malawi
		COI	MK033248	GenBank	Madagascar
Cx. atratus	Theobald, 1901	COI	MH376749	GenBank	Guadeloupe

		COI	MEDLI022-20	BOLD Systems	Guadeloupe
		ITS2	HQ317351	GenBank	USA
		ITS2	HQ317352	GenBank	USA
		ITS2	HQ317355	GenBank	USA
Cx. bitaeniorhynchus	Giles, 1901	COI	HQ398898	GenBank	Thailand
		COI	DQ154162	GenBank	India
		COI	AB690839	GenBank	Japan
		ITS2	DQ168421	GenBank	China
		ITS2	KY053484	GenBank	Sri Lanka
		ITS2	MF288839	GenBank	China
Cx. cedecei	Stone & Hair, 1968	COI	ACMIP141-07	BOLD Systems	USA
		COI	ACMIP144-07	BOLD Systems	USA
		COI	ACMIP137-07	BOLD Systems	USA
		ITS2	HQ317359	GenBank	USA
		ITS2	HQ317360	GenBank	USA
		ITS2	HQ317361	GenBank	USA
Cx. cinereus	Theobald, 1901	COI	MK533641	GenBank	Kenya
		COI	GQ165804	GenBank	Uganda
		COI	LC473616	GenBank	Malawi
Cx. corniger	Theobald, 1903	COI	FGMOS2266-20	BOLD Systems	French Guiana
		COI	MOSQV015-18	BOLD Systems	Mexico
		COI	KP281752	GenBank	Colombia
Cx. coronator	Dyar & Knab, 1906	COI	KX671386	GenBank	Brazil
		COI	MN997483	GenBank	Colombia
		COI	MT108602	GenBank	Mexico
		ITS2	GU299736	GenBank	Brazil
		ITS2	GU299742	GenBank	Brazil
		ITS2	GU562346	GenBank	USA
Cx. cylindricus	Theobald, 1903	COI	KU495009	GenBank	Australia
		COI	KU495010	GenBank	Australia
		COI	KU495013	GenBank	Australia
		ITS2	KU495645	GenBank	Australia
		ITS2	KX866001	GenBank	Australia
		ITS2	KX865990	GenBank	Australia
Cx. decens	Theobald, 1901	COI	MK033249	GenBank	Madagascar
		COI	MK033250	GenBank	Madagascar
Cx. duttoni	Theobald, 1901	COI	KU380363	GenBank	Kenya
		COI	LC473629	GenBank	Malawi
		COI	LC507861	GenBank	Ghana
Cx. erraticus	(Dyar & Knab, 1906)	COI	KM593022	GenBank	Colombia
		COI	KY859873	GenBank	Colombia
		COI	KX389306	GenBank	Canada
		ITS2	HQ317363	GenBank	USA
		ITS2	JF784424	GenBank	USA
		ITS2	U32861	GenBank	USA
Cx. foliatus	Brug, 1932	COI	MF278826	GenBank	China
Cx. gelidus	Theobald, 1901	COI	HQ398892	GenBank	Thailand
		COI	MF179172	GenBank	China
		COI	MF278815	GenBank	China
Cx. hayashii	Yamada, 1917	COI	LC054456	GenBank	Japan
		COI	LC104320	GenBank	Japan
		COI	MF278825	GenBank	China
Cx. hortensis	Ficalbi, 1889	COI	KJ012068	GenBank	Turkey
		COI	MK402673	GenBank	Spain
		COI	MN413913	GenBank	Spain
Cx. imitator	Theobald, 1903	COI	MF172282	GenBank	French Guiana
		COI	MF172283	GenBank	French Guiana
		COI	FGMOS1195-16	BOLD Systems	French Guiana
Cx. impudicus	Ficalbi, 1890	COI	KJ012081	GenBank	Turkey
		COI	MK402681	GenBank	Spain
		COI	KP037054	GenBank	Turkey
		28S	MT808438	GenBank	Thrace
Cx. iolambdis	Dyar, 1918	COI	ACMIP112-07	BOLD Systems	USA
		COI	ACMIP114-07	BOLD Systems	USA
		COI	ACMIP132-07	BOLD Systems	USA
		ITS2	HQ317367	GenBank	USA
		ITS2	HQ317368	GenBank	USA

		ITS2	HQ317369	GenBank	USA
Cx. mimulus	Edwards, 1915	COI	MF278820	GenBank	China
		COI	MG774467	GenBank	India
		COI	MH427577	GenBank	Laos
Cx. modestus	Ficalbi, 1890	COI	FM177758	GenBank	Russia
		COI	HF562836	GenBank	Germany
		COI	JN592723	GenBank	United Kingdom
		ITS2	KU880622	GenBank	China
		ITS2	MK972012	GenBank	United Kingdom
		ITS2	KU880623	GenBank	China
		28S	MT808439	GenBank	Thrace
Cx. neavei	Theobald, 1906	COI	LC473635	GenBank	Malawi
		COI	CULSA004-19	BOLD Systems	South Africa
Cx. nebulosus	Theobald, 1901	COI	KU187089	GenBank	Kenya
		COI	KU187090	GenBank	Kenya
		COI	KU187091	GenBank	Kenya
Cx. nigripalpus	Theobald, 1901	COI	JX259910	GenBank	Dominican Republic
		COI	KF919227	GenBank	Brazil
		COI	JQ957876	GenBank	Mexico
		ITS2	AF520974	GenBank	USA
		ITS2	GU299719	GenBank	Brazil
		ITS2	U33023	GenBank	USA
Cx. nigropunctatus	Edwards, 1926	COI	HQ398882	GenBank	Thailand
		COI	AY729976	GenBank	India
		COI	AB738106	GenBank	
Cx. peccator	Dyar & Knab, 1909	ITS2	HQ317375	GenBank	USA
		ITS2	HQ317376	GenBank	USA
		ITS2	HQ317377	GenBank	USA
Cx. perexiguus	Theobald, 1903	COI	KF406802	GenBank	Pakistan
		COI	KU380348	GenBank	Kenya
		COI	KJ012103	GenBank	Turkey
Cx. pilosus	(Dyar & Knab, 1906)	COI	FGMOS2096-20	BOLD Systems	French Guiana
		COI	FGMOS2209-20	BOLD Systems	French Guiana
		COI	KX779851	GenBank	Brazil
		ITS2	HQ317379	GenBank	USA
		ITS2	HQ317382	GenBank	USA
		ITS2	U33027	GenBank	USA
Cx. pipiens	Linnaeus, 1758	COI	CLX2.82; MPSAM009-21	Sampled	Bloemfontein Smallholding 2
		COI	CLX2.90; MPSAM010-21	Sampled	Bloemfontein Smallholding 2
		COI	CLX2.280; MPSAM014-21	Sampled	Bloemfontein Smallholding 2
		COI	CLX5.2.7; MPSAM021-21	Sampled	Gariep Dam Nature Reserve
		COI	CLX12.10; MPSAM034-21	Sampled	Memel
		COI	CLX21.10; MPSAM056-21	Sampled	Bloemfontein Urban Habitat 3
		COI	CLX22.42; MPSAM062-21	Sampled	Bloemfontein Semi-Pristine Habitat 3
		COI	JQ253847	GenBank	United Kingdom
		COI	AM403476	GenBank	Russia
		COI	FN395181	GenBank	Russia
		ITS2	CLX2.280; MPSAM014-21	Sampled	Bloemfontein Smallholding 2
		ITS2	CLX21.10; MPSAM056-21	Sampled	Bloemfontein Urban Habitat 3
		ITS2	DQ341109	GenBank	South Africa
		ITS2	U22115	GenBank	Laboratory Colony
		28S	X93384	GenBank	
		28S	MT808425	GenBank	Thrace
Cx. poicilipes	(Theobald, 1903)	COI	CULSA067-19	BOLD Systems	South Africa
		COI	LC473618	GenBank	Malawi
		COI	LC473619	GenBank	Malawi
Cx. portesi	Senevet & Abonnenc, 1941	COI	FGMOS2239-20	BOLD Systems	French Guiana

		COI	FGMOS2690-20	BOLD Systems	French Guiana
		COI	KX779852	GenBank	Brazil
		ITS2	MT151972	GenBank	Colombia
		ITS2	MT151985	GenBank	Colombia
Cx. quinquefasciatus	Say, 1823	COI	CLX4.2.19; MPSAM020-21	Sampled	Willem Pretorius Game Reserve
		COI	CLX13.5; MPSAM035-21	Sampled	Oranjeville
		COI	CLX18.2; MPSAM043-21	Sampled	Bloemfontein Urban Habitat 1
		COI	CLX19.2; MPSAM047-21	Sampled	Bloemfontein Urban Habitat 2
		COI	CLX21.5; MPSAM055-21	Sampled	Bloemfontein Urban Habitat 3
		COI	GQ165796	GenBank	Uganda
		COI	FN395201	GenBank	India
		COI	FM177756	GenBank	India
		ITS2	FJ416030	GenBank	Bangladesh
		ITS2	DQ168423	GenBank	China
		ITS2	GU299748	GenBank	Brazil
		28S	XR005279034	GenBank	South Africa
		28S	XR005279033	GenBank	South Africa
		28S	XR005279053	GenBank	South Africa
Cx. restuans	Theobald, 1901	COI	GU908085	GenBank	Canada
		COI	JX259912	GenBank	USA
		COI	KJ083844	GenBank	Canada
		ITS2	U22133	GenBank	USA
		ITS2	U22134	GenBank	USA
		ITS2	U22135	GenBank	USA
Cx. rima	Theobald, 1901	COI	LC473614	GenBank	Malawi
Cx. salinarius	Coquillett, 1904	COI	GU908096	GenBank	Canada
		COI	HQ944968	GenBank	USA
		COI	MH781016	GenBank	USA
		ITS2	U22139	GenBank	USA
		ITS2	U22140	GenBank	USA
		ITS2	U22141	GenBank	USA
Cx. salisburiensis	Theobald, 1901	COI	CLX9.2; MPSAM028-21	Sampled	Bethulie
Cx. simpsoni	Theobald, 1905	COI	KU187061	GenBank	Kenya
		COI	CULSA037-19	BOLD Systems	South Africa
Cx. spissipes	(Theobald, 1903)	COI	FGMOS1522-20	BOLD Systems	French Guiana
		COI	KX779868	GenBank	Brazil
		COI	KX779870	GenBank	Brazil
		ITS2	AY633736	GenBank	Venezuela
Cx. striatipes	Edwards, 1941	COI	KU187066	GenBank	Kenya
		COI	KU187065	GenBank	Kenya
Cx. tarsalis	Coquillett, 1896	COI	GU908102	GenBank	Canada
		COI	JX259917	GenBank	USA
		COI	JX297260	GenBank	Mexico
		ITS2	U33029	GenBank	USA
		ITS2	U33032	GenBank	USA
		ITS2	U33031	GenBank	USA
Cx. terzii	Edwards, 1941	COI	LC473644	GenBank	Malawi
Cx. theileri	Theobald, 1903	COI	CLX1.6; MPSAM001-21	Sampled	Bloemfontein Smallholding 1
		COI	CLX1.11; MPSAM002-21	Sampled	Bloemfontein Smallholding 1
		COI	CLX2.24; MPSAM006-21	Sampled	Bloemfontein Smallholding 2
		COI	CLX4.2.5; MPSAM018-21	Sampled	Willem Pretorius Game Reserve
		COI	CLX15.1; MPSAM037-21	Sampled	Boshof
		COI	FJ210898	GenBank	Iran
		COI	HE610457	GenBank	Portugal
		COI	JN051388	GenBank	Spain

		ITS2	CLX2.21; MPSAM005-21	Sampled	Bloemfontein Smallholding 2
		ITS2	CLX2.24; MPSAM006-21	Sampled	Bloemfontein Smallholding 2
		ITS2	CLX4.2.5; MPSAM018-21	Sampled	Willem Pretorius Game Reserve
		ITS2	JN051384	GenBank	Spain
		ITS2	KF483841	GenBank	Iran
		ITS2	MF288849	GenBank	China
		28S	CLX2.24; MPSAM006-21	Sampled	Bloemfontein Smallholding 2
Cx. torrentium	Martini, 1925	COI	AM403477	GenBank	Russia
		COI	FN395191	GenBank	Russia
		COI	CROCU050-20	BOLD Systems	Croatia
		ITS2	AJ850083	GenBank	Russia
		ITS2	MK972014	GenBank	United Kingdom
		ITS2	U33037	GenBank	Sweden / United Kingdom
Cx. trifoliatus	Edwards, 1914	COI	CULSA063-19	BOLD Systems	
Cx. tritaeniorhynchus	Giles, 1901	COI	HQ398885	GenBank	Thailand
		COI	DQ424952	GenBank	India
		ITS2	AF165896	GenBank	Japan
		ITS2	U33041	GenBank	Laboratory Colony
		ITS2	AF305558	GenBank	China
		28S	AF453490	GenBank	China
		28S	AF453493	GenBank	China
		28S	AF453489	GenBank	China
Cx. univittatus	Theobald, 1901	COI	CLX1.16; MPSAM003-21	Sampled	Bloemfontein Smallholding 1
		COI	CLX2.68; MPSAM007-21	Sampled	Bloemfontein Smallholding 2
		COI	CLX2.69; MPSAM008-21	Sampled	Bloemfontein Smallholding 2
		COI	CLX2.172; MPSAM011-21	Sampled	Bloemfontein Smallholding 2
		COI	CLX2.256; MPSAM013-21	Sampled	Bloemfontein Smallholding 2
		COI	CLX4.2.1; MPSAM017-21	Sampled	Willem Pretorius Game Reserve
		COI	CLX4.2.9; MPSAM019-21	Sampled	Willem Pretorius Game Reserve
		COI	CLX7.4; MPSAM023-21	Sampled	Rosendal
		COI	CLX8.3; MPSAM025-21	Sampled	Bethlehem
		COI	CLX9.4; MPSAM029-21	Sampled	Bethulie
		COI	CLX12.3; MPSAM032-21	Sampled	Memel
		COI	CLX12.5; MPSAM033-21	Sampled	Memel
		COI	CLX18.3; MPSAM044-21	Sampled	Bloemfontein Urban Habitat 1
		COI	GMSAB076-13	BOLD Systems	South Africa
		COI	KMPED801-18	BOLD Systems	South Africa
		COI	LC473638	GenBank	Malawi
		ITS2	CLX1.16; MPSAM003-21	Sampled	Bloemfontein Smallholding 1
		28S	CLX2.68; MPSAM007-21	Sampled	Bloemfontein Smallholding 2
		ITS2	CLX2.69; MPSAM008-21	Sampled	Bloemfontein Smallholding 2
		28S	CLX2.256; MPSAM013-21	Sampled	Bloemfontein Smallholding 2
		ITS2	CLX2.172; MPSAM011-21	Sampled	Bloemfontein Smallholding 2

		ITS2	CLX2.243; MPSAM012-21	Sampled	Bloemfontein Smallholding 2
		ITS2	CLX4.2.9; MPSAM019-21	Sampled	Willem Pretorius Game Reserve
		28S	CLX4.2.9; MPSAM019-21	Sampled	Willem Pretorius Game Reserve
Cx. vansomereni	Edwards, 1926	COI	KU380350	GenBank	Kenya
		COI	KU187047	GenBank	Kenya
		COI	KU187041	GenBank	Kenya
Cx. ybarmis	Dyar, 1920	COI	FGMOS2044-20	BOLD Systems	French Guiana
		ITS2	GU299688	GenBank	Brazil
		ITS2	GU299689	GenBank	Brazil
		Outgroup			
Cs. annulata	(Schrank, 1776)	COI	KM258141	GenBank	Belgium
		28S	MT808429	GenBank	
Cs. inconspicua	(Lee, 1937)	ITS2	KU495655	GenBank	Australia
Cs. longiareolata	(Macquart, 1838)	COI	KJ124849	GenBank	Austria
		28S	MT808423	GenBank	
Cs. melanura	(Coquillett, 1902)	ITS2	MH277929	GenBank	
		Anopheles			
		Ingroup			
An. aconitus	Dönitz, 1902	COI	DQ000253	GenBank	Thailand
		COI	HQ877378	GenBank	Vietnam
		COI	AY423055	GenBank	Vietnam
		ITS2	AJ626945	GenBank	Thailand
		ITS2	AF194491	GenBank	Thailand
		ITS2	AY547360	GenBank	Thailand
An. albimanus	Wiedemann, 1820	COI	KU892037	GenBank	Colombia
		COI	KU892036	GenBank	Colombia
		COI	KY921770	GenBank	Colombia
		ITS2	HM042300	GenBank	Ecuador
		ITS2	GU477267	GenBank	Colombia
		ITS2	JX212820	GenBank	Panama
		28S	L78065	GenBank	
An. albitarsis	Lynch Arribálzaga, 1878	COI	JQ615234	GenBank	Brazil
		COI	JF923680	GenBank	Brazil
		COI	DQ076204	GenBank	Argentina
		ITS2	AF462385	GenBank	Brazil
		ITS2	U92332	GenBank	
		ITS2	AY828320	GenBank	Brazil
		28S	JQ697082	GenBank	Brazil
An. annularis	van der Wulp, 1884	COI	KF406653	GenBank	Pakistan
		COI	MH535929	GenBank	India
		COI	JN832671	GenBank	India
		ITS2	EF192273	GenBank	
		ITS2	EU366358	GenBank	India
		ITS2	DQ279445	GenBank	India
An. argyritarsis	Robineau-Desvoidy, 1827	COI	JF923685	GenBank	Brazil
		COI	KU671369	GenBank	Brazil
		COI	KU892050	GenBank	Colombia
		ITS2	U92347	GenBank	
		ITS2	U92356	GenBank	
		ITS2	HM022413	GenBank	Colombia
An. atacamensis	González & Sallum, 2010	COI	JF923686	GenBank	Chile
		COI	GQ902966	GenBank	Chile
		COI	MF381600	GenBank	Brazil
		ITS2	GQ902959	GenBank	Chile
		ITS2	GQ902961	GenBank	Chile
		ITS2	GQ902964	GenBank	Chile
An. balabacensis	Baisas, 1936	COI	MH032606	GenBank	Malaysia
		COI	MG002524	GenBank	Malaysia
		COI	MG002536	GenBank	Malaysia
		ITS2	MG008613	GenBank	Malaysia

		ITS2	MT623038	GenBank	Malaysia
		ITS2	KY883198	GenBank	Malaysia
An. bancroftii	Giles, 1902	COI	MN733776	GenBank	New Caledonia
		COI	MN733775	GenBank	New Caledonia
		ITS2	AF203380	GenBank	Australia / Papua New Guinea
		ITS2	MF716525	GenBank	New Caledonia
		ITS2	MN733813	GenBank	New Caledonia
An. barbirostris	van der Wulp, 1884	COI	AB435996	GenBank	Thailand
		COI	HM773367	GenBank	India
		COI	AB331570	GenBank	Thailand
		ITS2	AB331551	GenBank	Thailand
		ITS2	KJ462242	GenBank	Malaysia
		ITS2	KC878682	GenBank	China
An. braziliensis	(Chagas, 1907)	COI	JF923690	GenBank	Brazil
		COI	KC555062	GenBank	Venezuela
		COI	MH844114	GenBank	Brazil
		ITS2	AF461753	GenBank	Brazil
		ITS2	KP994156	GenBank	French Guiana
		ITS2	HM022412	GenBank	Colombia
An. cinereus	Theobald, 1901	ITS2	MN460356	GenBank	Ethiopia
		ITS2	KM068090	GenBank	Saudi Arabia
An. claviger	(Meigen, 1804)	COI	HE614028	GenBank	
		COI	KM258237	GenBank	Belgium
		COI	JX255719	GenBank	Tajikistan
		ITS2	AY129232	GenBank	
		ITS2	KP749460	GenBank	Italy
		ITS2	DQ229313	GenBank	Iran
		28S	MT808441	GenBank	Thrace
An. coustani	Laveran, 1900	COI	MK585965	GenBank	Mali
		COI	KJ522831	GenBank	Kenya
		COI	KM097001	GenBank	Guinea-Bissau
		ITS2	MK129245	GenBank	Madagascar
		ITS2	JN994134	GenBank	Zambia
		ITS2	KJ522815	GenBank	Kenya
An. culicifacies	Giles, 1901	COI	GQ259182	GenBank	India
An. culicifacies	Giles, 1901	COI	DQ424962	GenBank	India
An. culicifacies	Giles, 1901	COI	AY917198	GenBank	India
An. culicifacies	Giles, 1901	ITS2	KY000682	GenBank	Sri Lanka
An. culicifacies	Giles, 1901	ITS2	AY007168	GenBank	China
An. culicifacies	Giles, 1901	ITS2	EF192274	GenBank	
An. cydippis / squamosus	De Meillon, 1931 / Theobald, 1901	COI	ANO7.1; MPSAM022-21	Sample	Rosendal
		COI	ANO22.1; MPSAM061-21	Sample	Bloemfontein Semi-Pristine Habitat 3
An. darlingi	Root, 1926	COI	MH924379	GenBank	Colombia
		COI	HM022406	GenBank	Colombia
		COI	KC894593	GenBank	Colombia
		ITS2	U92337	GenBank	
		ITS2	HM022434	GenBank	Colombia
		ITS2	AF032133	GenBank	Brazil
An. demeilloni	Evans, 1933	COI	LC473587	GenBank	Malawi
		COI	LC473589	GenBank	Malawi
		COI	LC473588	GenBank	Malawi
		ITS2	MW257148	GenBank	Kenya
		ITS2	MW257149	GenBank	Kenya
		ITS2	MW257150	GenBank	Kenya
An. dthali	Patton, 1905	COI	KM068084	GenBank	Saudi Arabia
		COI	KM068074	GenBank	Saudi Arabia
		COI	KM068073	GenBank	Saudi Arabia
		ITS2	DQ662408	GenBank	Iran
		ITS2	JF966736	GenBank	Iran
		ITS2	AY445827	GenBank	Iran
An. epiroticus	Linton & Harbach, 2005 (in Linton *et al.*, 2005)	COI	AY789182	GenBank	Southeast Asia
		COI	AY789187	GenBank	Southeast Asia
		COI	AY789184	GenBank	Southeast Asia

		ITS2	AF469855	GenBank	Thailand
		ITS2	AY662406	GenBank	Vietnam
		ITS2	AY662407	GenBank	Vietnam
		28S	MW078488	GenBank	Myanmar
		28S	MW078489	GenBank	India
An. farauti	Laveran, 1902	COI	KM016080	GenBank	Tajikistan
		COI	KM016079	GenBank	Tajikistan
		COI	KF202340	GenBank	Solomon Archipelago
		ITS2	EF042696	GenBank	Papua New Guinea
		ITS2	HM584365	GenBank	Papua New Guinea
		ITS2	AF055984	GenBank	Australia
An. flavirostris	(Ludlow, 1914)	COI	MT753041	GenBank	Indonesia
		COI	MT753040	GenBank	Indonesia
		ITS2	GQ500121	GenBank	East Timor
		ITS2	GU062188	GenBank	Timor-Leste
		ITS2	AY943666	GenBank	Philippines
An. fluviatilis	James, 1902	COI	DQ154155	GenBank	India
		COI	DQ154158	GenBank	India
		COI	DQ154156	GenBank	India
		ITS2	AY049001	GenBank	Iran
		ITS2	AF440788	GenBank	Iran
		ITS2	AF167298	GenBank	India
An. fluviatilis S	James, 1902	28S	DQ523565	GenBank	
An. fluviatilis T	James, 1902	28S	DQ523566	GenBank	
An. funestus	Giles, 1900	COI	KU380404	GenBank	Kenya
		COI	MK300232	GenBank	Kenya
		COI	MH299890	GenBank	Kenya
		ITS2	AY035721	GenBank	Madagascar
		ITS2	JN994135	GenBank	Zambia
		ITS2	AF062512	GenBank	Africa
		28S	AF171046	GenBank	Africa
An. gambiae	Giles, 1902	COI	LC507832	GenBank	Ghana
		COI	KU187108	GenBank	Kenya
		COI	KR152326	GenBank	Togo
		ITS2	AF307110	GenBank	Benin
		ITS2	EU104634	GenBank	Angola
		ITS2	AF470163	GenBank	Gambia
		28S	KC177663	GenBank	
		28S	BX042283	GenBank	
An. homunculus	Komp, 1937	COI	JQ291239	GenBank	Brazil
		COI	JQ291235	GenBank	Brazil
		COI	JQ291233	GenBank	Brazil
		ITS2	FJ176945	GenBank	Brazil
		ITS2	JQ291243	GenBank	Brazil
		ITS2	DQ364655	GenBank	Brazil
An. jeyporiensis	James, 1902	COI	DQ154157	GenBank	India
		COI	GQ259195	GenBank	India
		COI	JN881334	GenBank	India
		ITS2	HQ873038	GenBank	India
		ITS2	JX289827	GenBank	India
		ITS2	AF230466	GenBank	Vietnam
An. kochi	Dönitz, 1901	COI	MK893403	GenBank	Thailand
		COI	MK893404	GenBank	Thailand
		COI	MT669942	GenBank	Malaysia
		ITS2	EU650424	GenBank	China
		ITS2	MK881135	GenBank	Thailand
		ITS2	MT623059	GenBank	Malaysia
An. kompi	Edwards, 1930	COI	JF923715	GenBank	Brazil
		COI	NC_037827	GenBank	Brazil
		COI	MF537257	GenBank	Brazil
An. koreicus	Yamada & Watanabe, 1918	COI	LC054417	GenBank	Japan
		COI	LC054416	GenBank	Japan
		COI	LC054415	GenBank	Japan
		ITS2	MW546413	GenBank	South Korea
		ITS2	AY523635	GenBank	China
		ITS2	DQ177500	GenBank	South Korea

		28S	MW546416	GenBank	South Korea
		28S	MW546413	GenBank	South Korea
An. leesoni	Evans, 1931	COI	KJ522840	GenBank	Kenya
		COI	KR014852	GenBank	Zambia
		COI	AY423056	GenBank	South Africa
		ITS2	JN994139	GenBank	Zambia
		ITS2	KJ522824	GenBank	Kenya
		ITS2	KR014834	GenBank	Zambia
An. lindesayi	Giles, 1900	COI	MH294483	GenBank	India
		COI	MH190218	GenBank	India
		COI	MH330153	GenBank	India
		ITS2	MT076994	GenBank	Bhutan
		ITS2	MW546420	GenBank	South Korea
		ITS2	AJ620898	GenBank	
		28S	MW546425	GenBank	South Korea
		28S	MW546420	GenBank	South Korea
An. longipalpis	(Theobald, 1903)	COI	MH547427	GenBank	Kenya
		COI	KR014848	GenBank	Zambia
An. longipalpis C	(Koekemoer *et al*., 2009)	COI	MH547426	GenBank	Kenya
		ITS2	EF136463	GenBank	Zambia
		ITS2	MH536653	GenBank	Kenya
		ITS2	KR014831	GenBank	Zambia
An. longirostris	Brug, 1928	ITS2	GU170434	GenBank	Papua New Guinea
		ITS2	GU170535	GenBank	Papua New Guinea
		ITS2	JF717381	GenBank	Papua New Guinea
An. longirostris A	Brug, 1928	COI	GU247056	GenBank	Papua New Guinea
		COI	GU247055	GenBank	Papua New Guinea
		COI	GU247054	GenBank	Papua New Guinea
An. maculatus	Theobald, 1901	COI	MK685248	GenBank	
		COI	JN596972	GenBank	India
		COI	DQ267690	GenBank	India
		ITS2	AF500072	GenBank	Malaysia
		ITS2	FJ526581	GenBank	Cambodia
		ITS2	AY491974	GenBank	Malaysia
An. maculipalpis	Giles, 1902	COI	KJ522833	GenBank	Kenya
		COI	MH384978	GenBank	Democratic Republic of the Congo
		COI	KR014851	GenBank	Zambia
		ITS2	MK129244	GenBank	Madagascar
		ITS2	JN994142	GenBank	Zambia
		ITS2	KJ522817	GenBank	Kenya
An. maculipennis	Meigen, 1818	COI	KM258229	GenBank	Belgium
		COI	AF342716	GenBank	Greece
		COI	DQ118176	GenBank	Greece
		ITS2	AF436065	GenBank	Iran
		ITS2	AF455818	GenBank	Greece
		ITS2	AF455853	GenBank	Greece
An. marshallii	(Theobald, 1903)	ITS2	MW257137	GenBank	Cameroon
		ITS2	MW257138	GenBank	Cameroon
		ITS2	MW257139	GenBank	Cameroon
An. merus	Dönitz, 1902	COI	MG560163	GenBank	
		COI	KT382824	GenBank	
		ITS2	GQ870313	GenBank	Tanzania
An. minimus	Theobald, 1901	COI	AY917196	GenBank	India
		COI	GQ259181	GenBank	India
		COI	HQ398936	GenBank	Vietnam
		ITS2	AB088355	GenBank	Thailand
		ITS2	GQ871783	GenBank	India
		ITS2	JN975434	GenBank	Japan
An. minimus A	Theobald, 1901	28S	DQ523567	GenBank	
An. multicolor	Cambouliu, 1902	ITS2	AY564228	GenBank	Iran
		ITS2	AY564229	GenBank	Iran
		ITS2	MT790357	GenBank	Mauritania
An. nili	(Theobald, 1904)	ITS2	MH598416	GenBank	Guinea
		ITS2	KR014820	GenBank	Zambia
		ITS2	KC189966	GenBank	Cameroon
An. nuneztovari	Gabaldon, 1940	COI	HQ315876	GenBank	Colombia

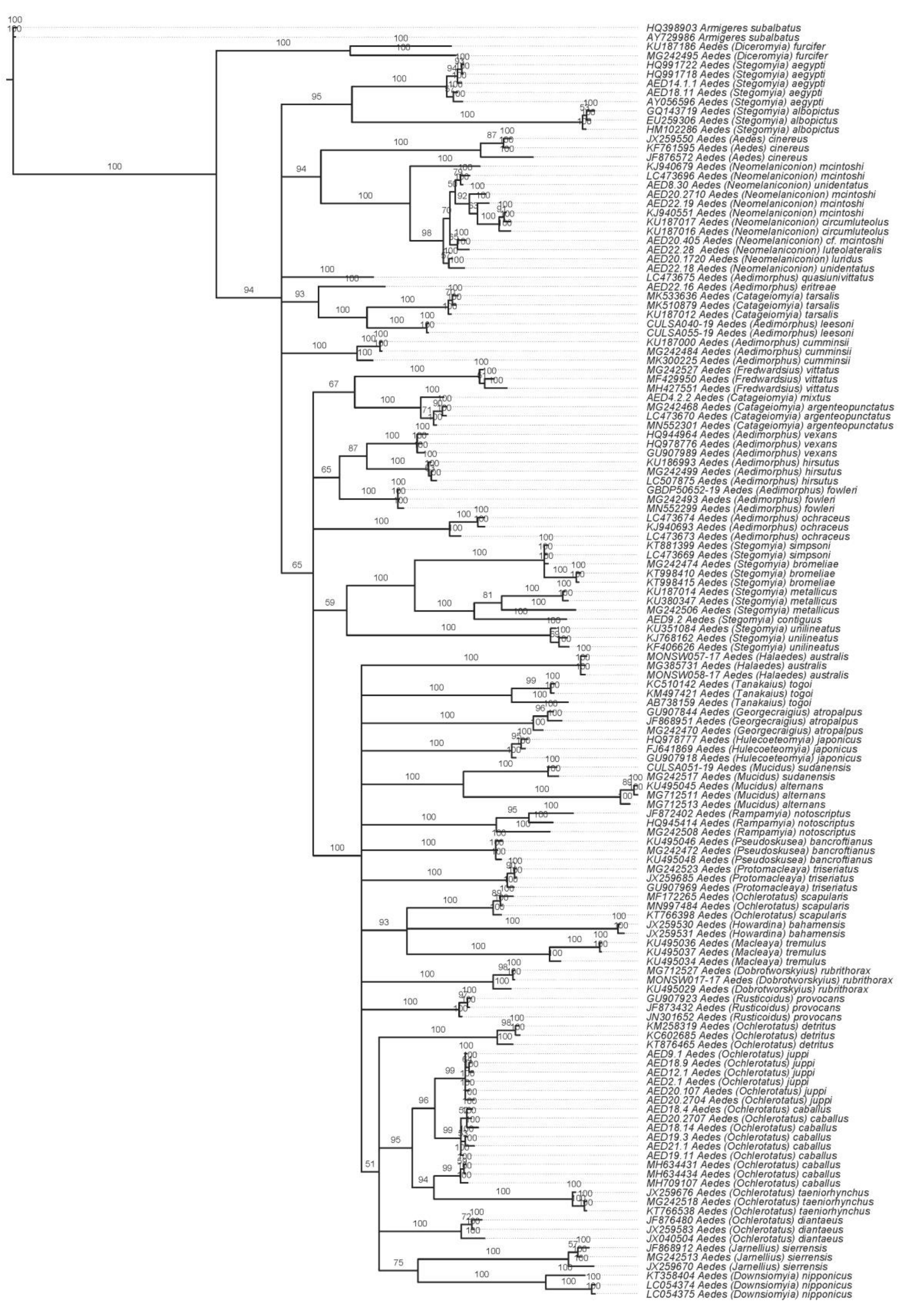
		COI	KU865542	GenBank	Brazil
		COI	JF923710	GenBank	Brazil
		ITS2	AF461749	GenBank	Brazil
		ITS2	AY028081	GenBank	Colombia
		ITS2	HQ020390	GenBank	Colombia
An. parensis	Gillies, 1962	COI	AY423061	GenBank	Kenya
		COI	JQ424713	GenBank	South Africa
		COI	JQ424738	GenBank	South Africa
		ITS2	AY035720	GenBank	Africa
		ITS2	JN994144	GenBank	Zambia
An. parvus	(Chagas, 1907)	ITS2	JN023051	GenBank	Brazil
		ITS2	JN023064	GenBank	Brazil
		ITS2	JN023065	GenBank	Brazil
An. pharoensis	Theobald, 1901	COI	KR014844	GenBank	Zambia
		COI	KU380430	GenBank	Kenya
		COI	MK585980	GenBank	Mali
		ITS2	MT366211	GenBank	Belgium
		ITS2	MT408572	GenBank	Kenya
		ITS2	KR014827	GenBank	Zambia
An. plumbeus	Stephens, 1828	COI	MK402728	GenBank	Spain
		COI	KM258215	GenBank	Belgium
		COI	MH463068	GenBank	
		ITS2	MK625347	GenBank	Italy
		ITS2	KC294435	GenBank	Iran
		ITS2	AJ555483	GenBank	Russia
An. pretoriensis	(Theobald, 1903)	COI	KJ522836	GenBank	Kenya
		COI	MK628501	GenBank	Ethiopia
		COI	KR014846	GenBank	Zambia
		ITS2	JN994145	GenBank	Zambia
		ITS2	KJ522820	GenBank	Kenya
		ITS2	KR014829	GenBank	Zambia
An. pseudopunctipennis	Theobald, 1901	COI	KY921775	GenBank	Colombia
		COI	KC354819	GenBank	Colombia
		COI	HM022408	GenBank	Colombia
		ITS2	HM022428	GenBank	Colombia
		ITS2	KC354810	GenBank	Colombia
		ITS2	KF436937	GenBank	Colombia
An. punctimacula	Dyar & Knab, 1906	COI	MN997532	GenBank	Colombia
		COI	KF698833	GenBank	Colombia
		COI	KU900757	GenBank	Colombia
		ITS2	GU477275	GenBank	Colombia
		ITS2	JX212806	GenBank	Panama
		ITS2	KF698889	GenBank	Colombia
An. punctipennis	(Say, 1823)	COI	GU908015	GenBank	Canada
		COI	KR653634	GenBank	Canada
		COI	JX259786	GenBank	USA
		ITS2	MN460678	GenBank	USA
		ITS2	MN460780	GenBank	USA
		ITS2	MN460781	GenBank	USA
An. rivulorum	Leeson, 1935	COI	KR014839	GenBank	Zambia
		COI	AY423060	GenBank	South Africa
		COI	JQ424739	GenBank	South Africa
		ITS2	JN994148	GenBank	South Africa
		ITS2	KR014822	GenBank	Zambia
		ITS2	AF180524	GenBank	Kenya
An. rufipes	(Gough, 1910)	COI	MK586044	GenBank	Mali
		COI	KJ522838	GenBank	Kenya
		COI	MK586051	GenBank	Mali
		ITS2	MK129242	GenBank	Madagascar
		ITS2	JN994150	GenBank	Zambia
		ITS2	KR014828	GenBank	Zambia
An. saperoi	Bohart & Ingram, 1946	COI	AB738176	GenBank	
		COI	AB738292	GenBank	
		COI	AB738150	GenBank	
		ITS2	AY425324	GenBank	Japan
		ITS2	AY425331	GenBank	Japan

		ITS2	AY425328	GenBank	Japan
An. sergentii	(Theobald, 1907)	COI	MK353402	GenBank	Egypt
		COI	KR152335	GenBank	Egypt
		COI	KR152336	GenBank	Egypt
		ITS2	MF631763	GenBank	Morocco
		ITS2	KT160246	GenBank	Egypt
		ITS2	AY533851	GenBank	Iran
An. sinensis	Wiedemann, 1828	COI	AB738166	GenBank	
		COI	GQ265918	GenBank	South Korea
		COI	GU908045	GenBank	South Korea
		ITS2	AF543861	GenBank	Laboratory colony
		ITS2	AY130469	GenBank	South Korea
		ITS2	AJ004942	GenBank	China
		28S	GU384700	GenBank	China
		28S	MW546421	GenBank	South Korea
		28S	GU384693	GenBank	South Korea
Anopheles sp.		COI	ANO3.1; MPSAM015-21	Sample	Bloemfontein Semi-Pristine Habitat 1
		ITS2	ANO3.1; MPSAM015-21	Sample	Bloemfontein Semi-Pristine Habitat 1
An. splendidus	Koidzumi, 1920	COI	AY917207	GenBank	India
		COI	KF406678	GenBank	Pakistan
		COI	EU256339	GenBank	India
		ITS2	EF192276	GenBank	
		ITS2	MF535235	GenBank	China
		ITS2	FJ526600	GenBank	Cambodia
An. squamosus	Theobald, 1901	COI	MK586052	GenBank	Mali
		COI	KJ522841	GenBank	Kenya
		COI	LC473607	GenBank	Malawi
		ITS2	MK592071	GenBank	Africa
		ITS2	KJ522825	GenBank	Kenya
		ITS2	KR014825	GenBank	Zambia
An. stephensi	Liston, 1901	COI	FJ210893	GenBank	Iran
		COI	GU908046	GenBank	South Africa
		COI	DQ154166	GenBank	India
		ITS2	AY157678	GenBank	Iran
		ITS2	AY365049	GenBank	Iran
		ITS2	AY702482	GenBank	Iran
An. subpictus	Grassi, 1899 (in Grassi *et al.*, 1899)	COI	KC970277	GenBank	India
		COI	DQ267688	GenBank	India
		COI	DQ310146	GenBank	India
		ITS2	AF406613	GenBank	Sri Lanka
		ITS2	EF601868	GenBank	India
		ITS2	EU847232	GenBank	
		28S	MW078487	GenBank	South Asia
		28S	MW078485	GenBank	South Asia
		28S	MW078484	GenBank	South Asia
An. sundaicus	(Rodenwaldt, 1925)	COI	AY672395	GenBank	Indonesia
		COI	GU908047	GenBank	Malaysia
		COI	AY789711	GenBank	Malaysia
		ITS2	AF369550	GenBank	Malaysia
		ITS2	AY662245	GenBank	Malaysia
		ITS2	GQ284820	GenBank	Vietnam
		28S	MW078490	GenBank	South Asia
An. superpictus	Grassi, 1899	COI	JX255706	Sample	Tajikistan
		COI	JX255707	GenBank	Tajikistan
		COI	JX255705	GenBank	Tajikistan
		ITS2	AY515163	GenBank	Uzbekistan
		ITS2	DQ487138	GenBank	Iran
		ITS2	AY941109	GenBank	Iran
		28S	MT808443	GenBank	Thrace
An. tenebrosus	Dönitz, 1902	COI	KU187097	GenBank	Kenya
		COI	KU187096	GenBank	Kenya
An. tessellatus	Theobald, 1901	COI	AB738146	GenBank	
		COI	MF179266	GenBank	China
		COI	MK685249	GenBank	

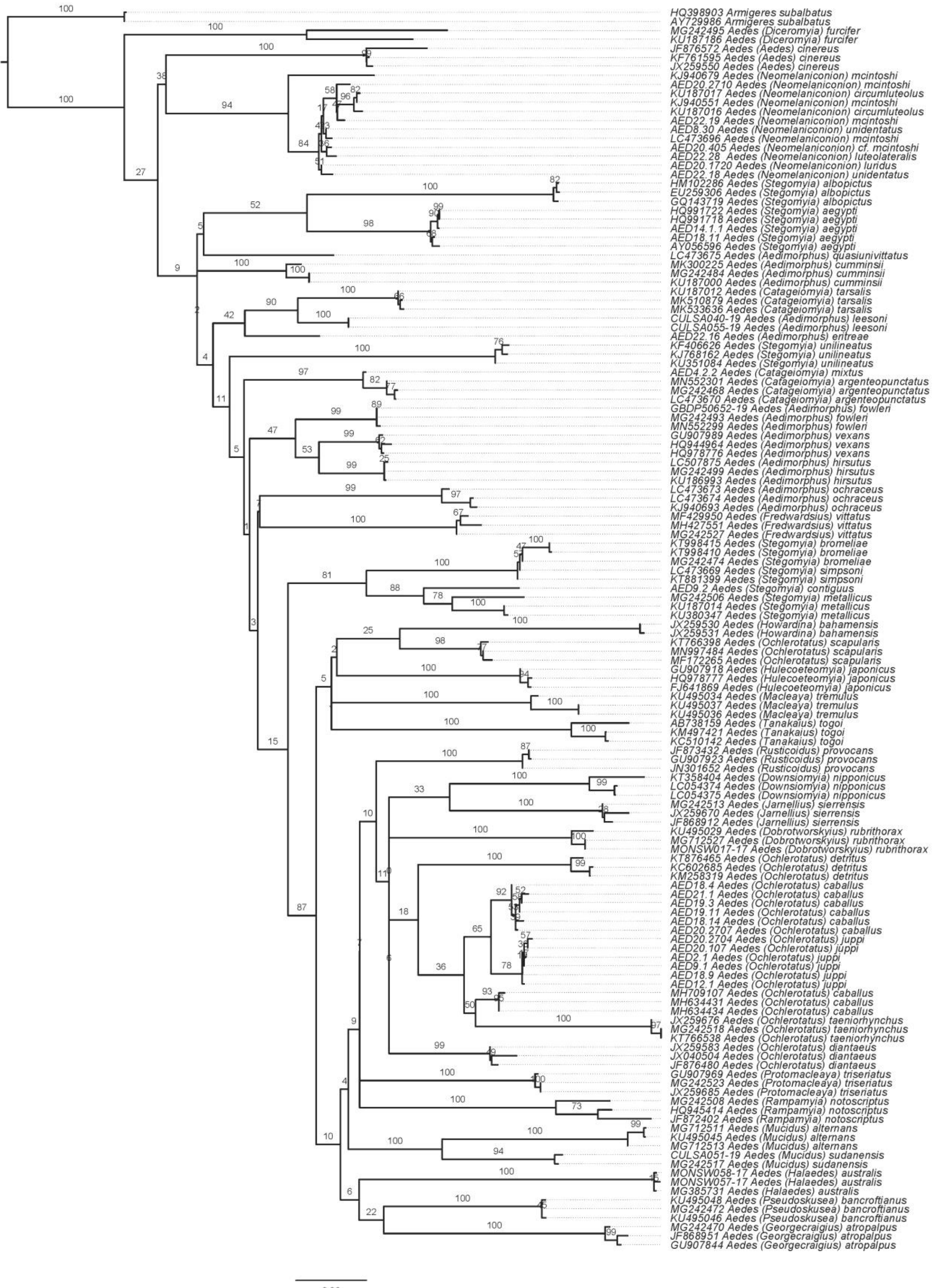
		ITS2	MN203103	GenBank	Indonesia
		ITS2	EU650425	GenBank	China
		ITS2	MT623072	GenBank	Malaysia
An. theileri	Edwards, 1912	ITS2	MH378771	GenBank	Democratic Republic of the Congo
		ITS2	JN994151	GenBank	Zambia
		ITS2	KR014821	GenBank	Zambia
An. triannulatus	(Neiva & Pinto, 1922)	COI	JF923736	GenBank	Brazil
		COI	JF923733	GenBank	Brazil
		COI	HM022387	GenBank	Colombia
		ITS2	AF462377	GenBank	Brazil
		ITS2	HM022422	GenBank	Colombia
		ITS2	U92331	GenBank	
An. vagus	Dönitz, 1902	COI	MH425411	GenBank	Vietnam
		COI	JQ915196	GenBank	India
		COI	MF179259	GenBank	China
		ITS2	EU919718	GenBank	China
		ITS2	FJ457631	GenBank	China
		ITS2	FJ654648	GenBank	Southeast Asia
		Outgroup			
Tx. ambionensis	(Doleschall, 1857)	ITS2	U48377	GenBank	
		28S	KC177664	GenBank	
		28S	FJ040569	GenBank	
Tx. haemorrhoidalis	(Fabricius, 1787)	COI	MF172394	GenBank	French Guiana
Toxorhynchites sp.		ITS2	MF537256	GenBank	Brazil
Tx. splendens	(Wiedemann, 1819)	COI	HQ398877	GenBank	Thailand

5.5 APPENDIX 5

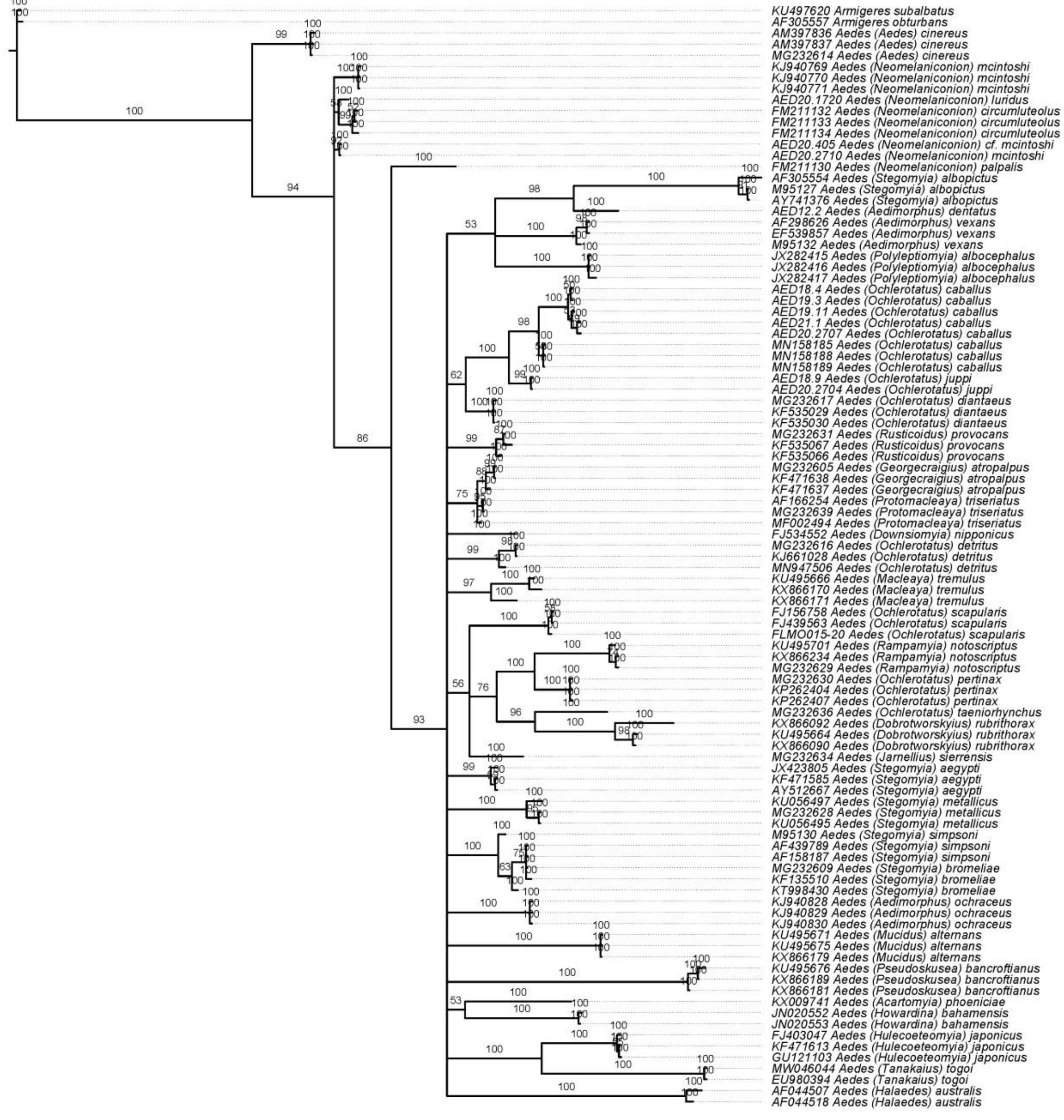
5.5.1 *AEDES*, COI, BAYESIAN ANALYSIS



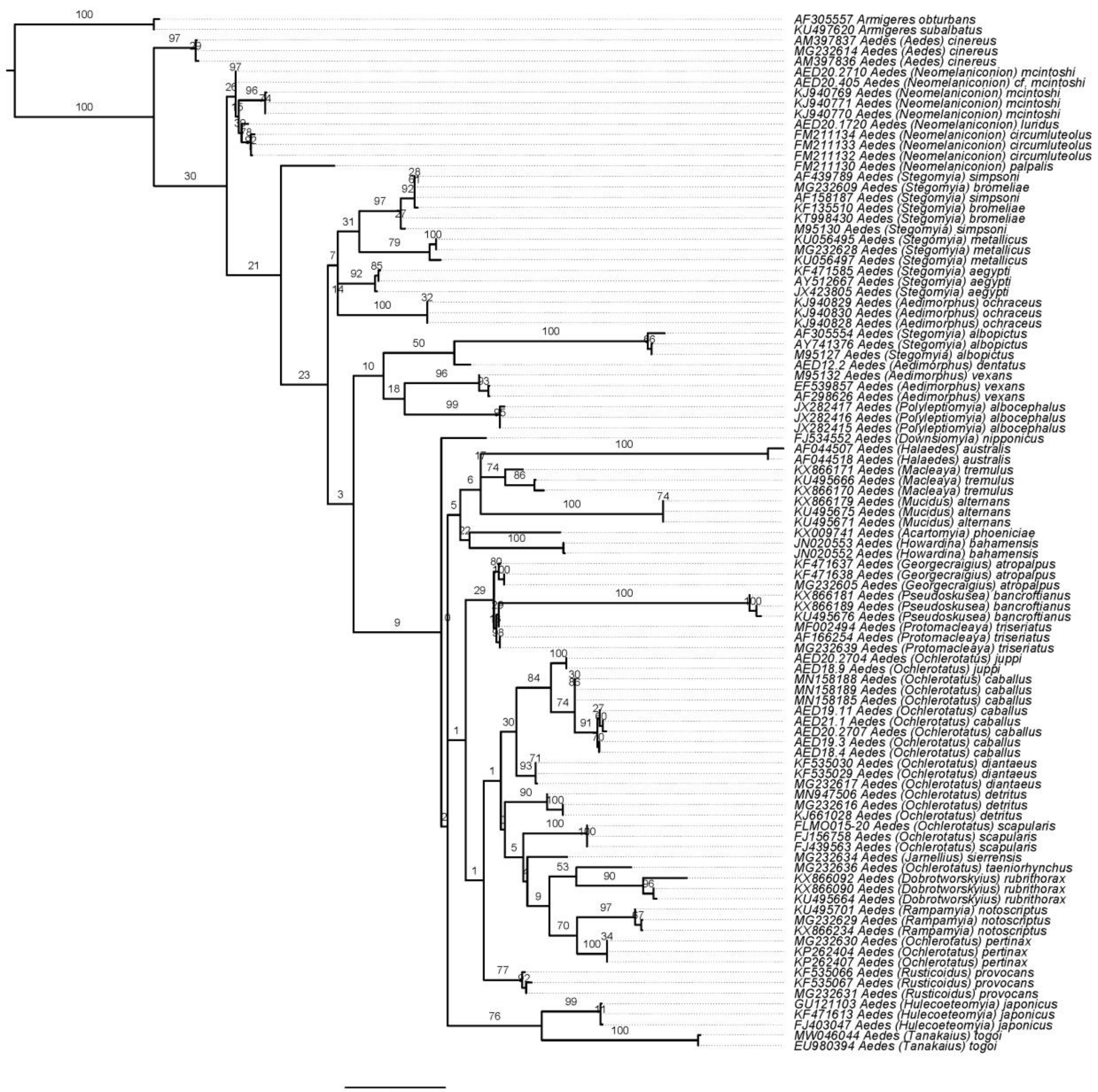
5.5.2 *AEDES*, COI, MAXIMUM LIKELIHOOD ANALYSIS



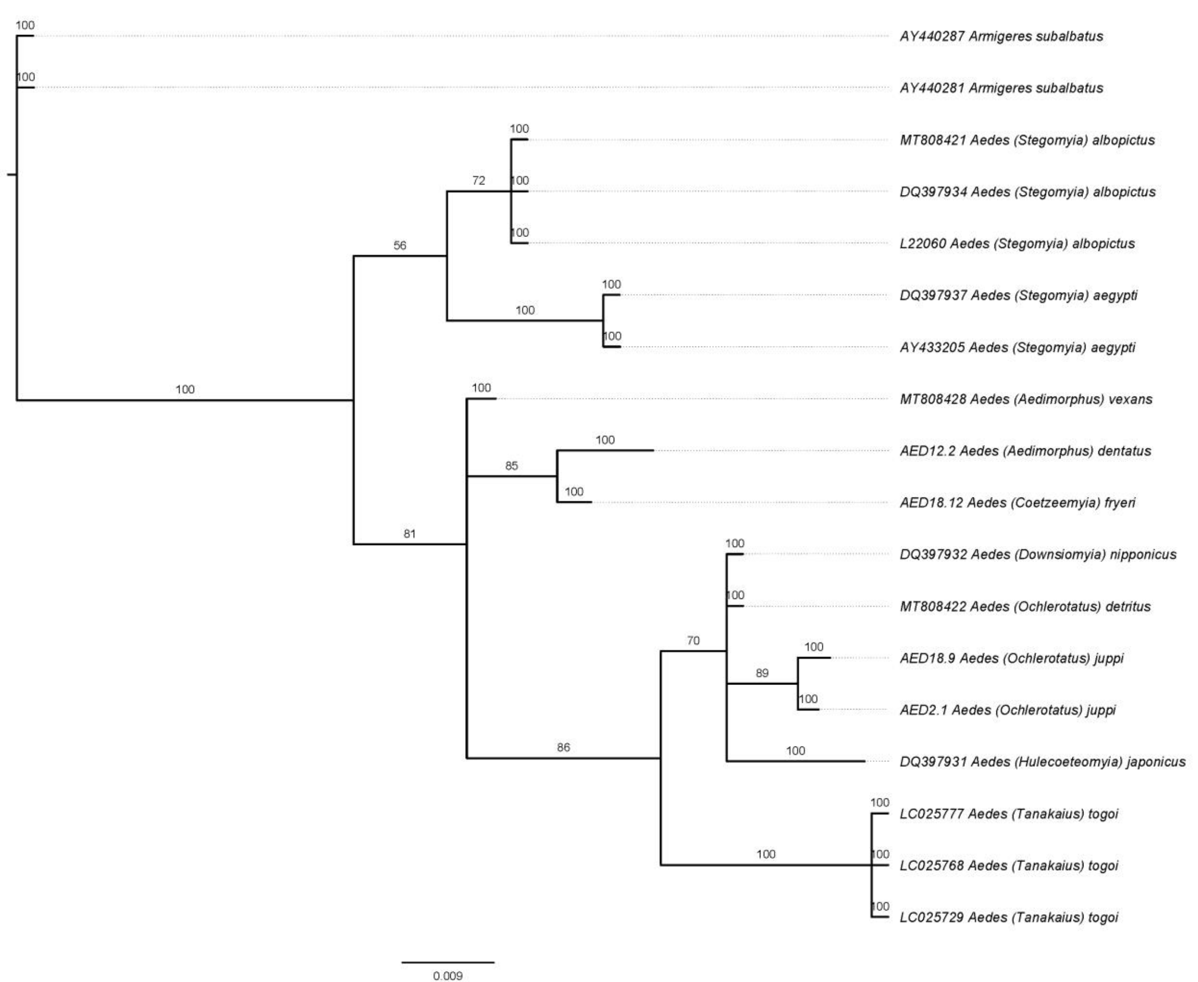
5.5.3 *AEDES*, ITS2, BAYESIAN ANALYSIS



5.5.4 *AEDES*, ITS2, MAXIMUM LIKELIHOOD ANALYSIS



5.5.5 *AEDES*, 28S, BAYESIAN ANALYSIS



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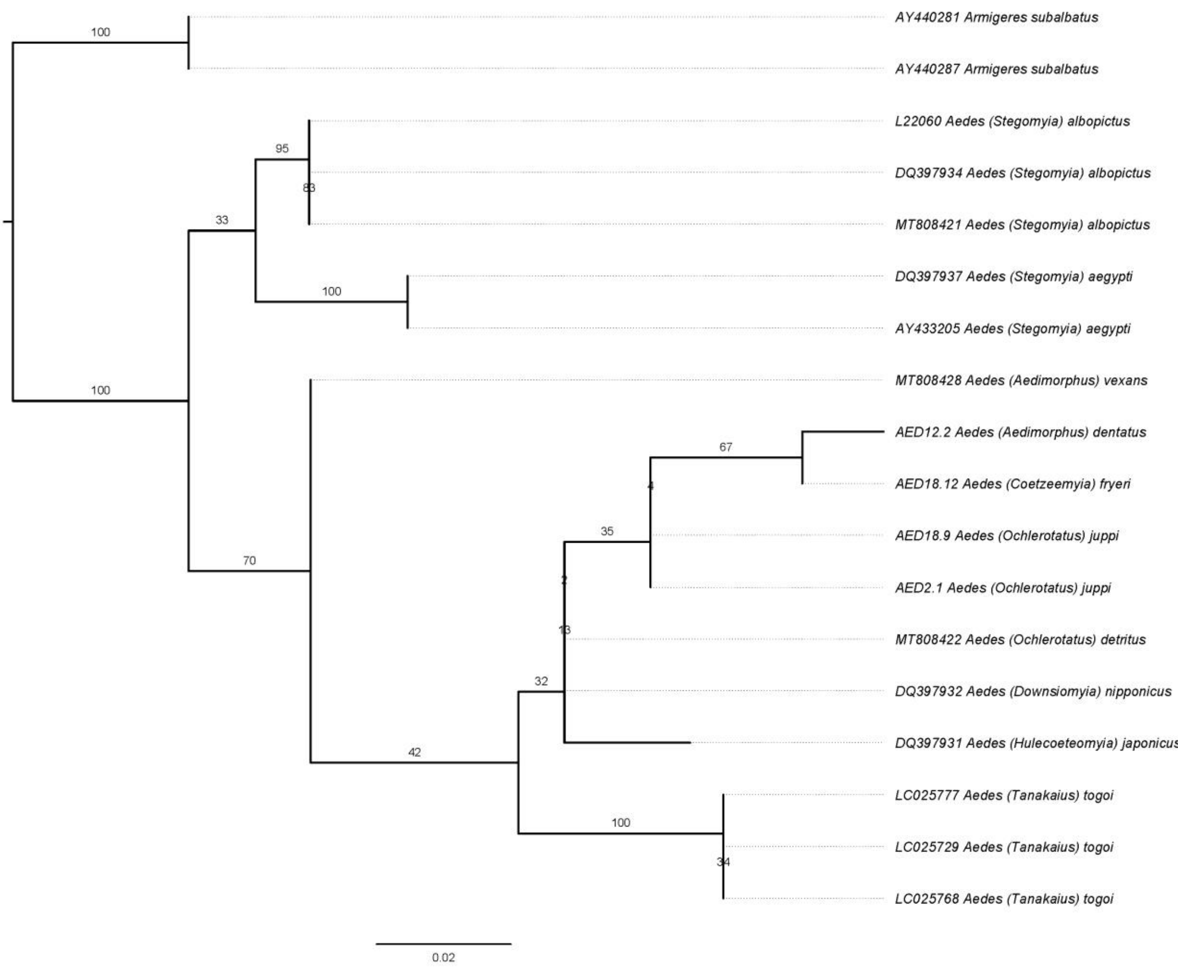
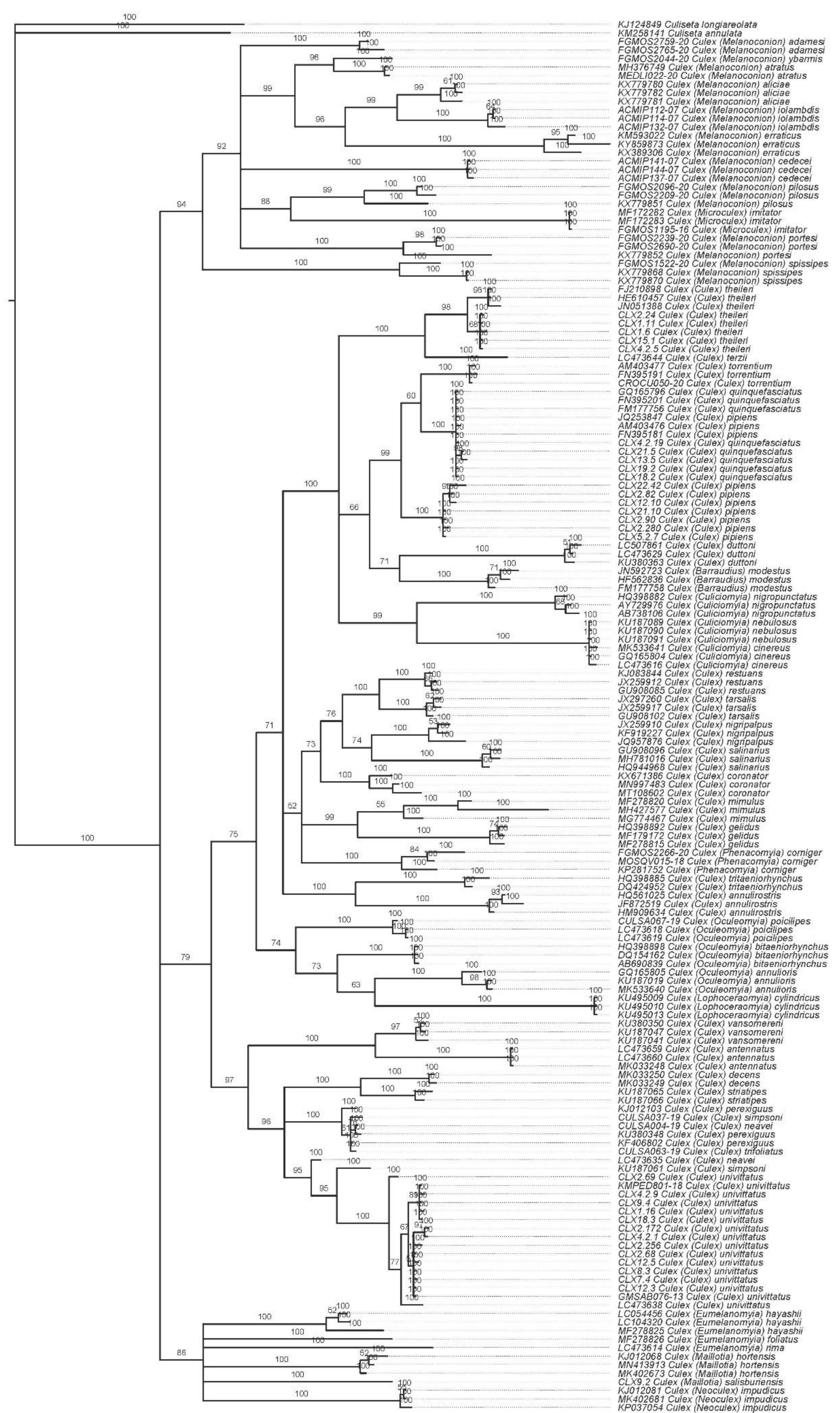
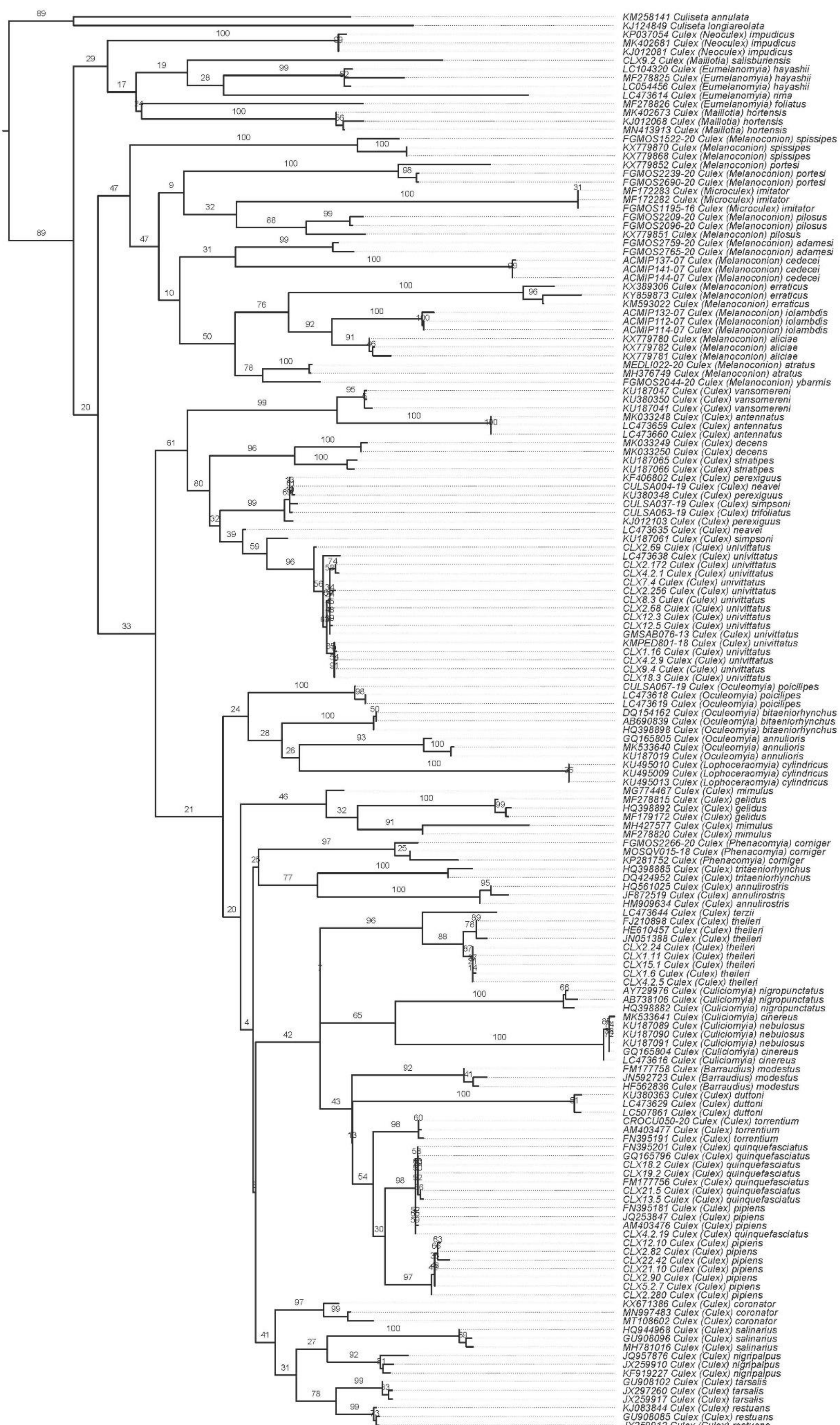


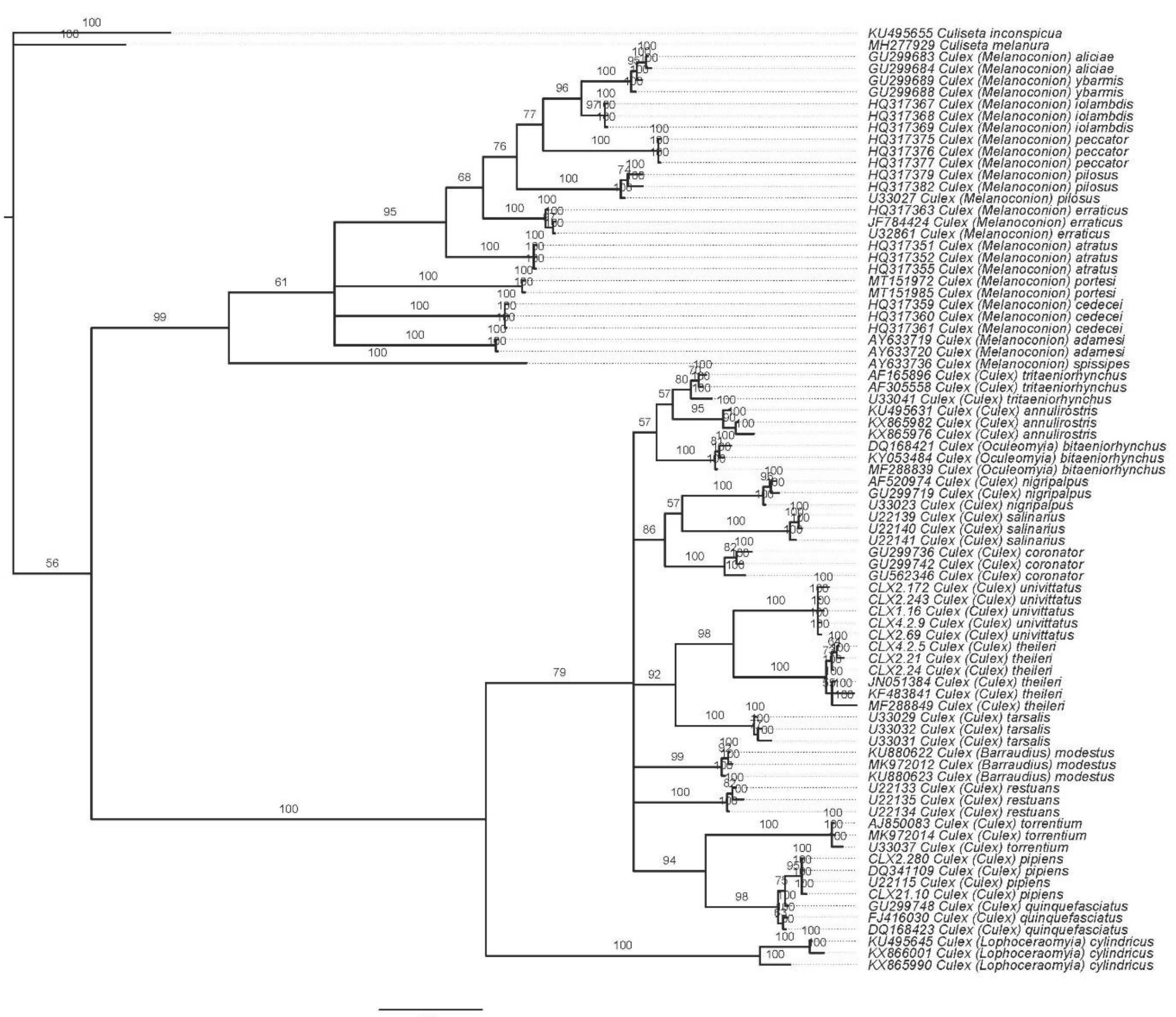
5.5.7 *CULEX*, COI, BAYESIAN ANALYSIS



5.5.8 *CULEX*, COI, MAXIMUM LIKELIHOOD ANALYSIS



5.5.9 *CULEX*, ITS2, BAYESIAN ANALYSIS



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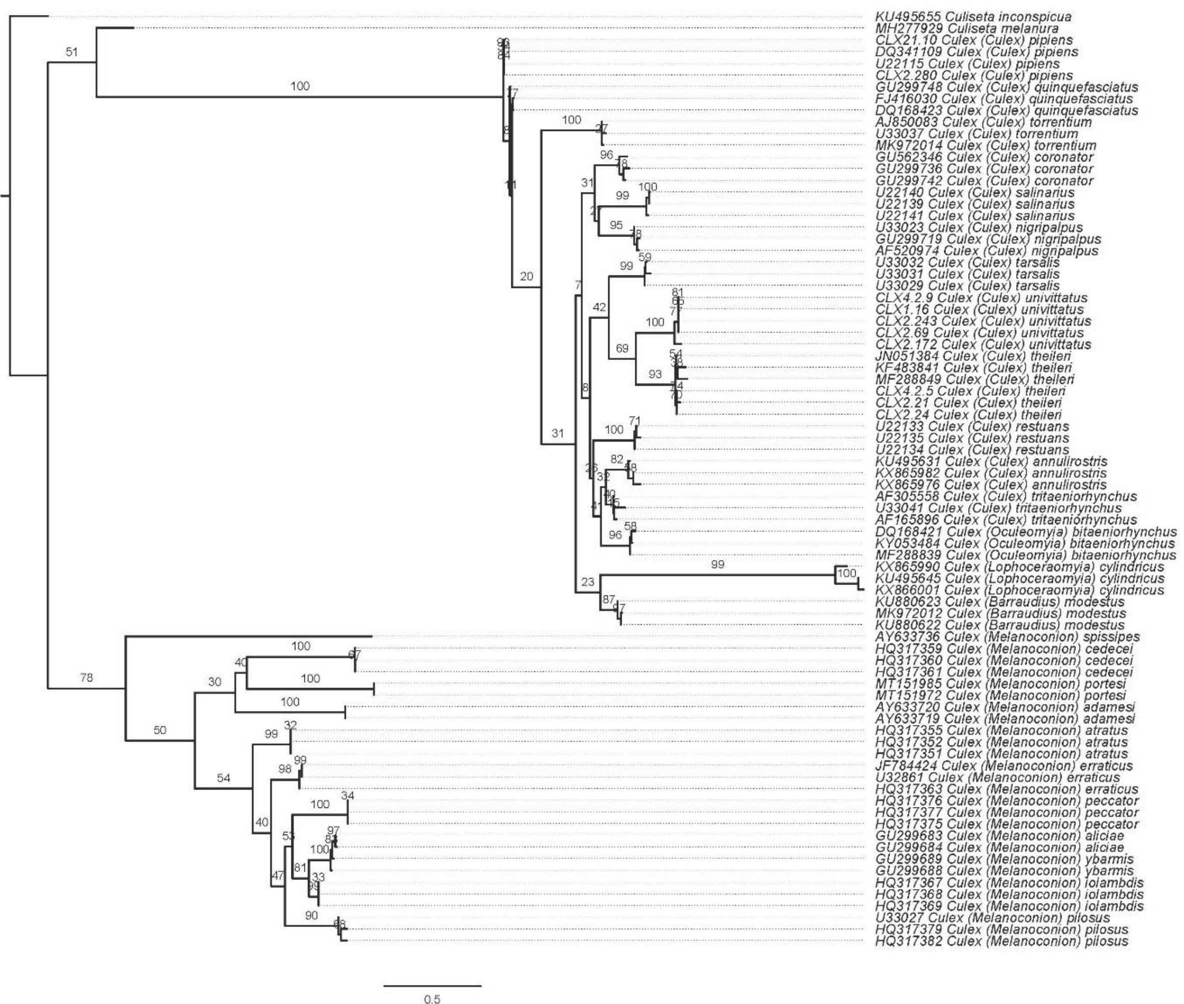
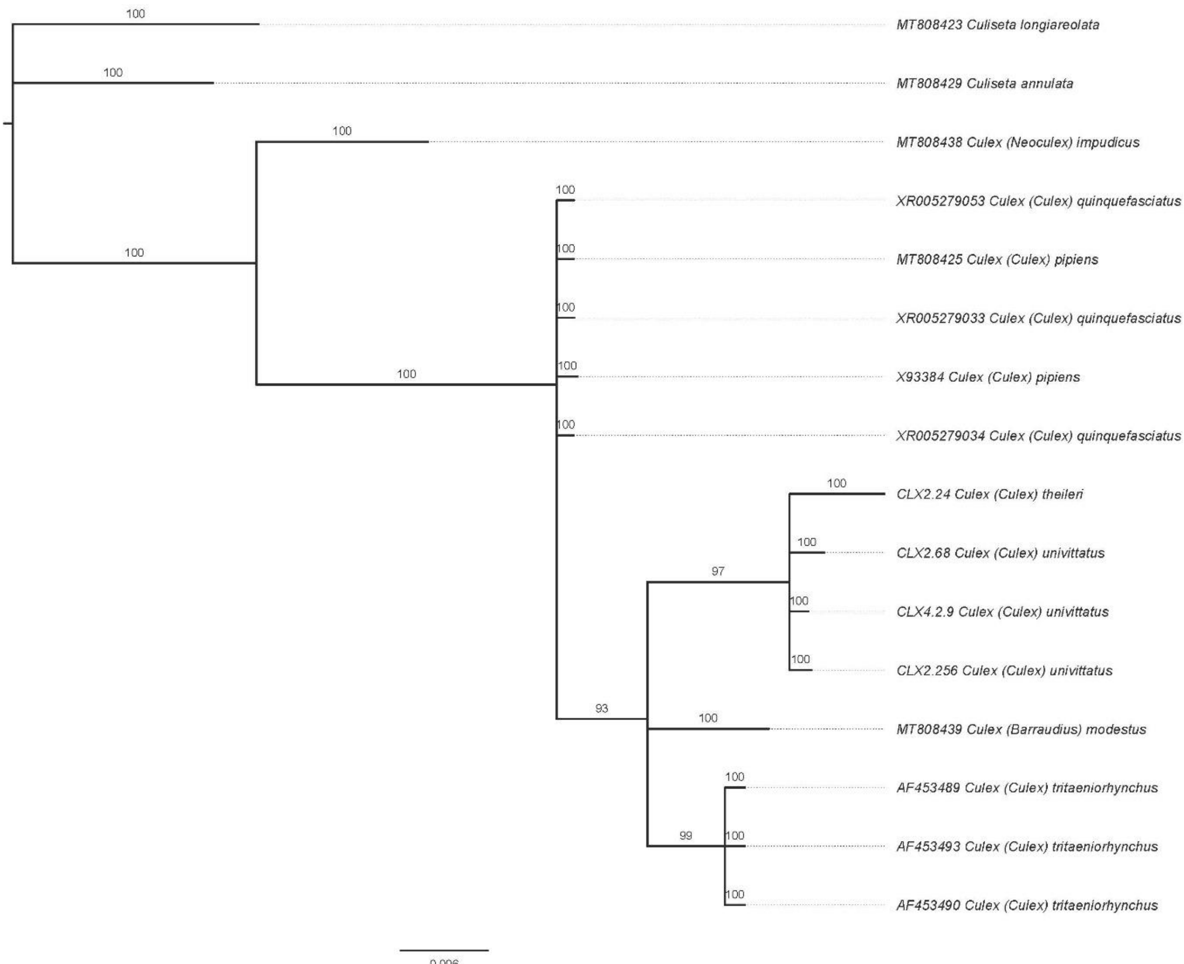
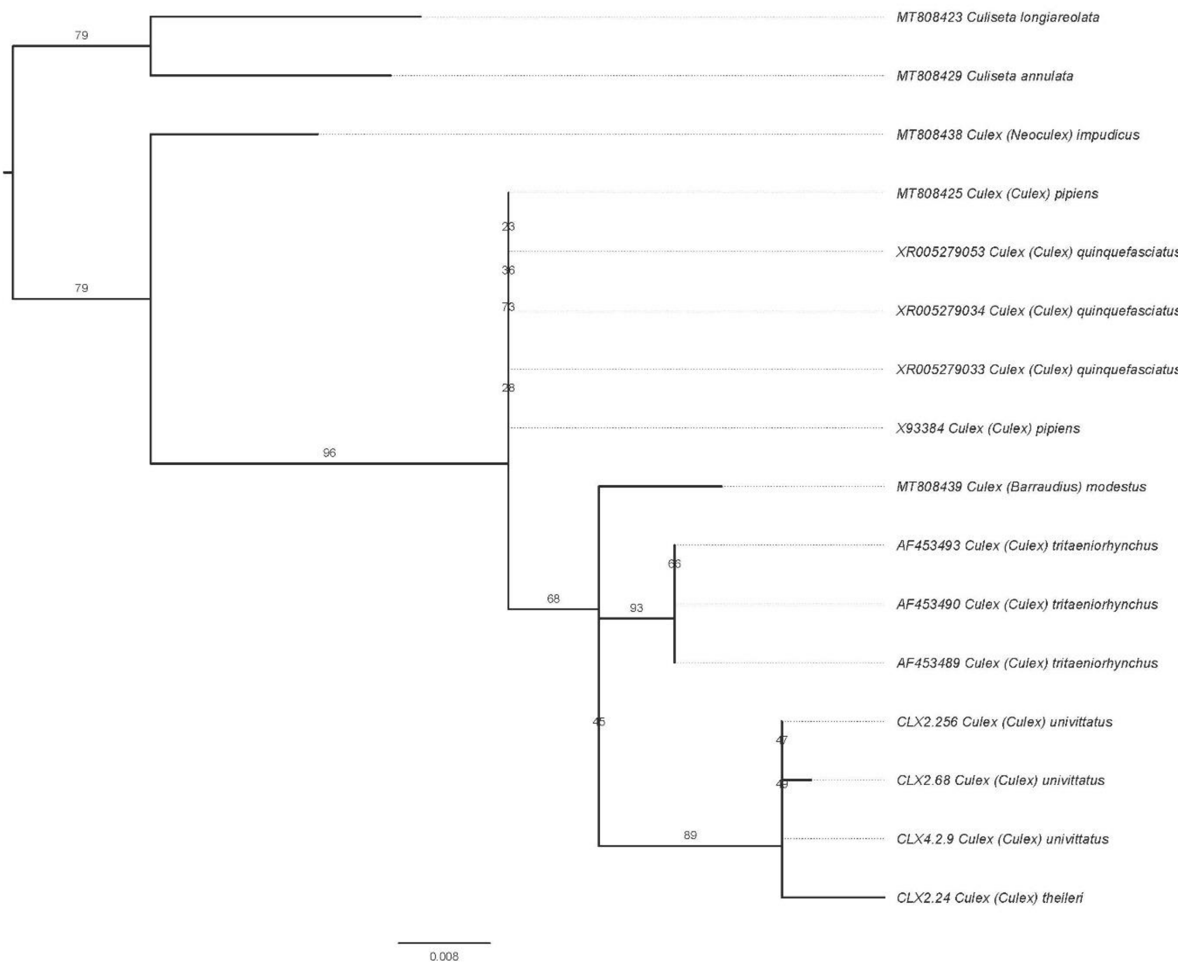


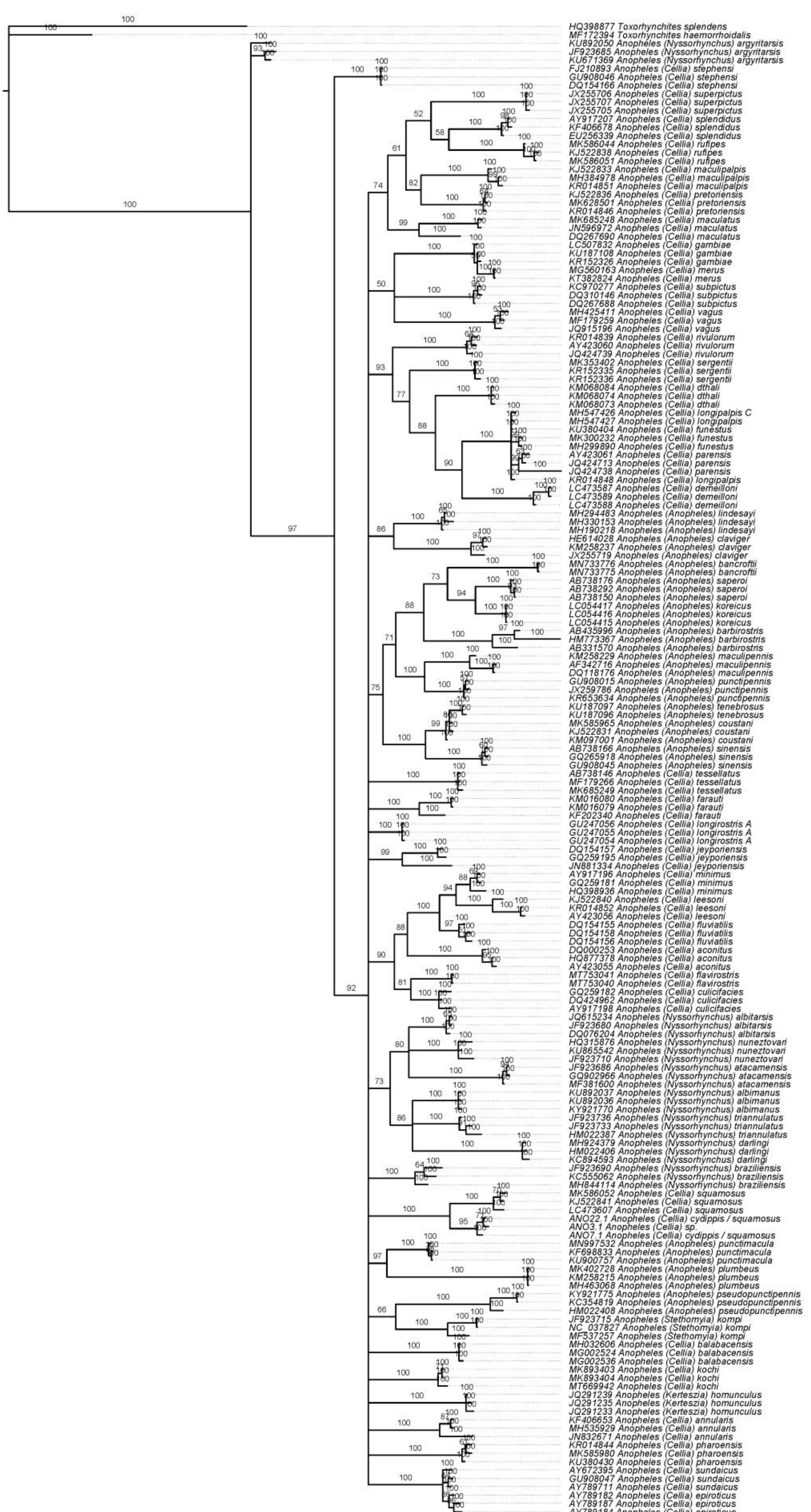
5.5.11 *CULEX*, 28S, BAYESIAN ANALYSIS



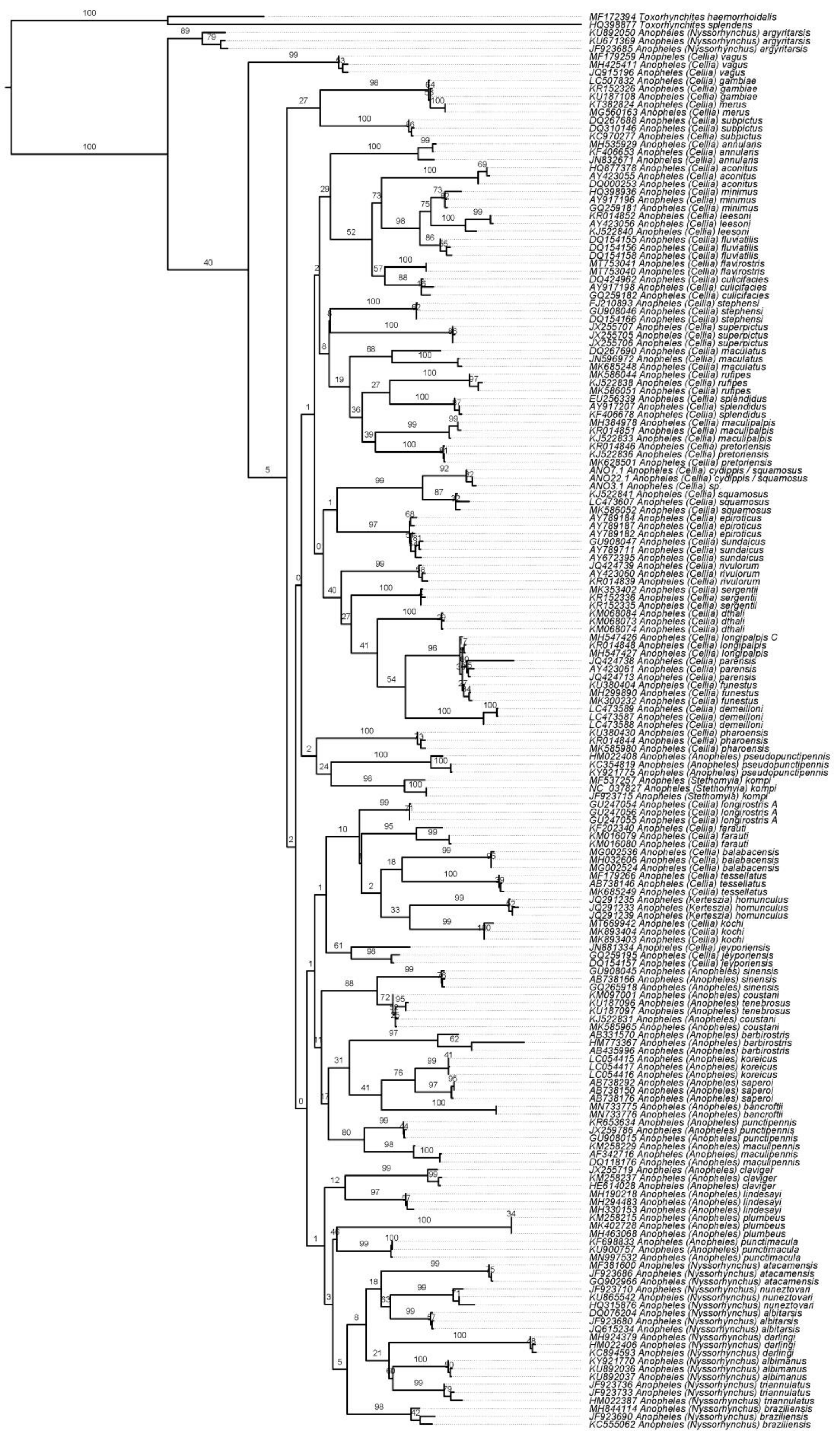
5.5.12 *CULEX*, 28S, MAXIMUM LIKELIHOOD ANALYSIS



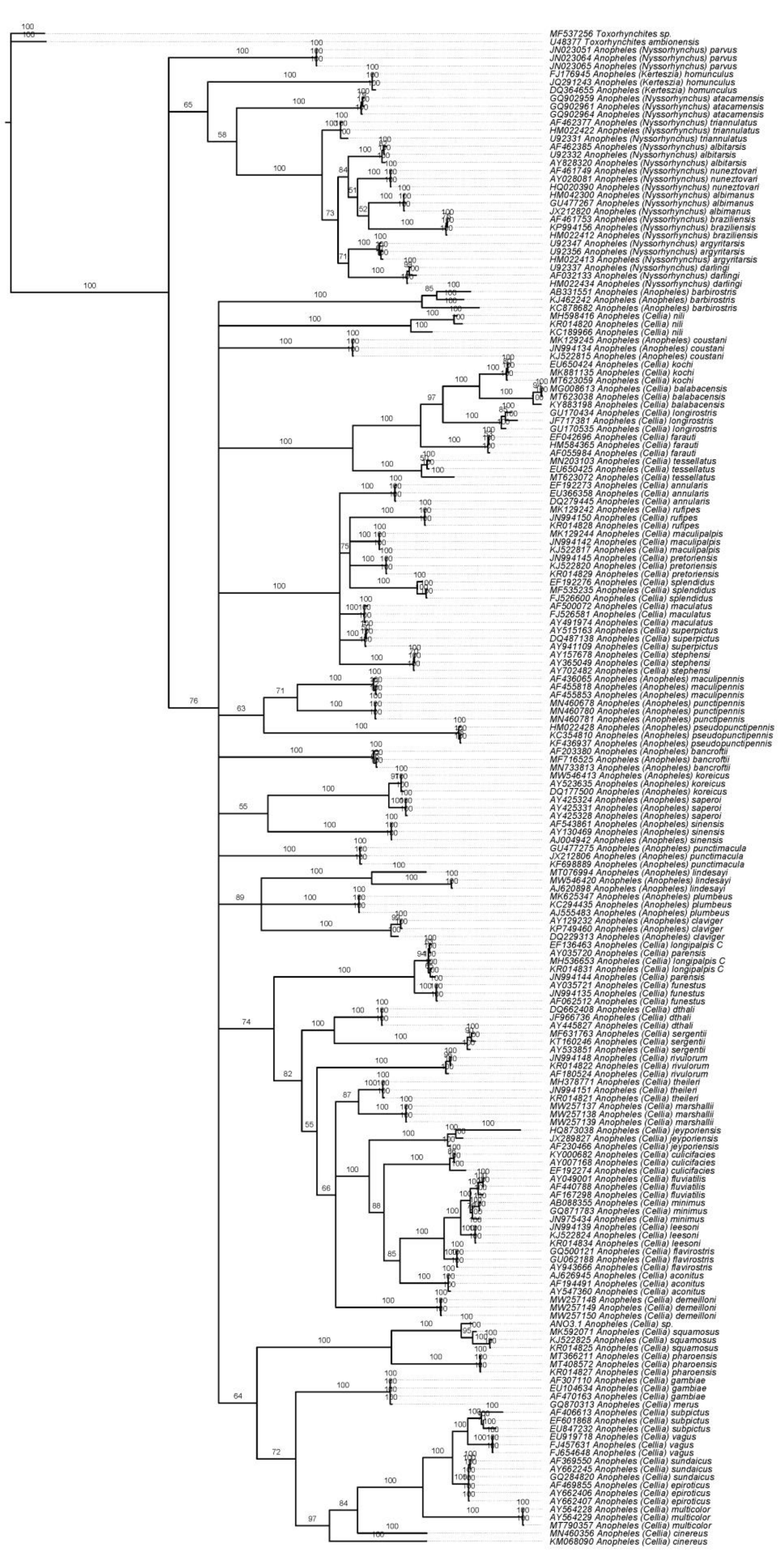
5.5.13 *ANOPHELES*, COI, BAYESIAN ANALYSIS



5.5.14 *ANOPHELES*, COI, MAXIMUM LIKELIHOOD ANALYSIS



5.5.15 *ANOPHELES*, ITS2, BAYESIAN ANALYSIS



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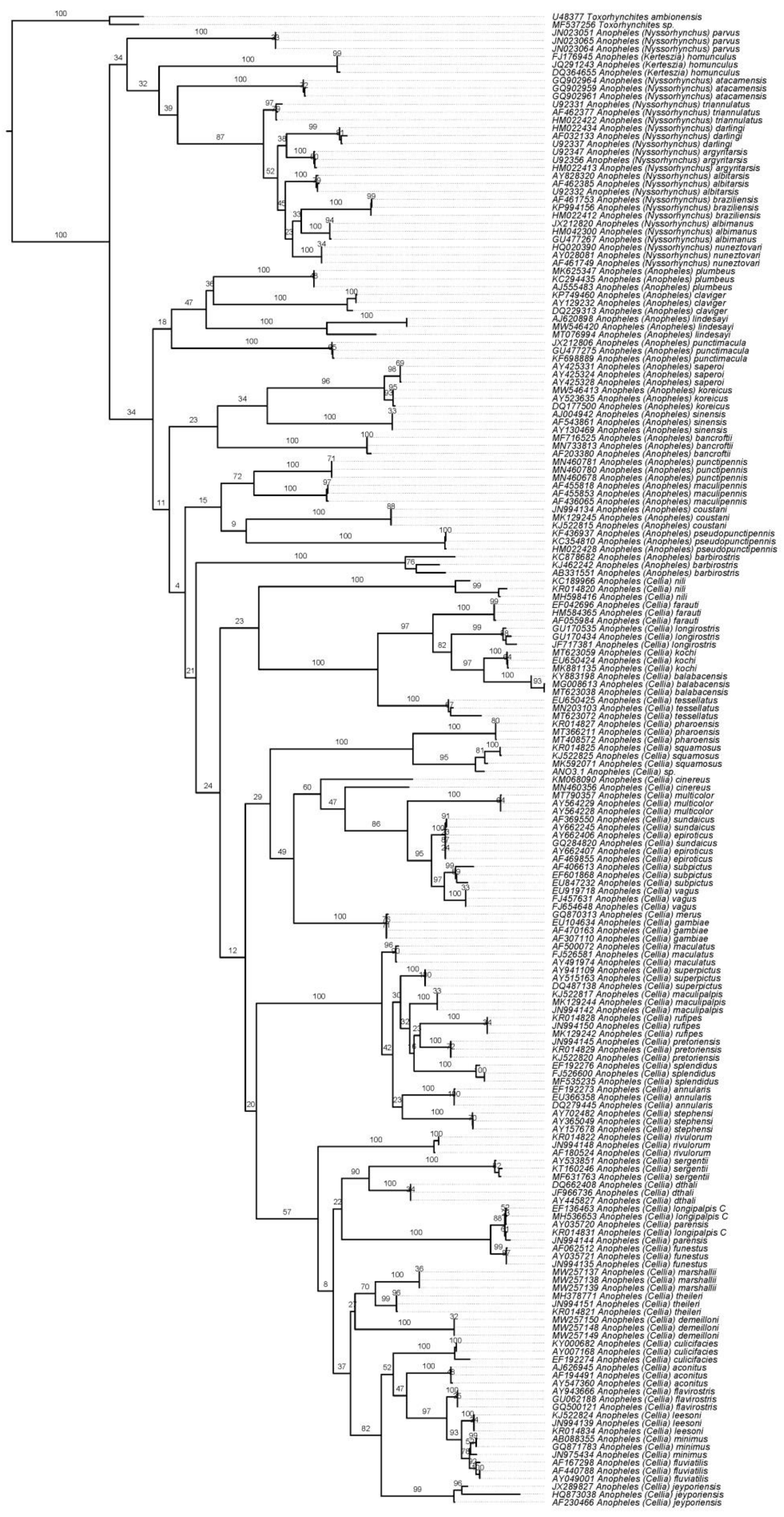
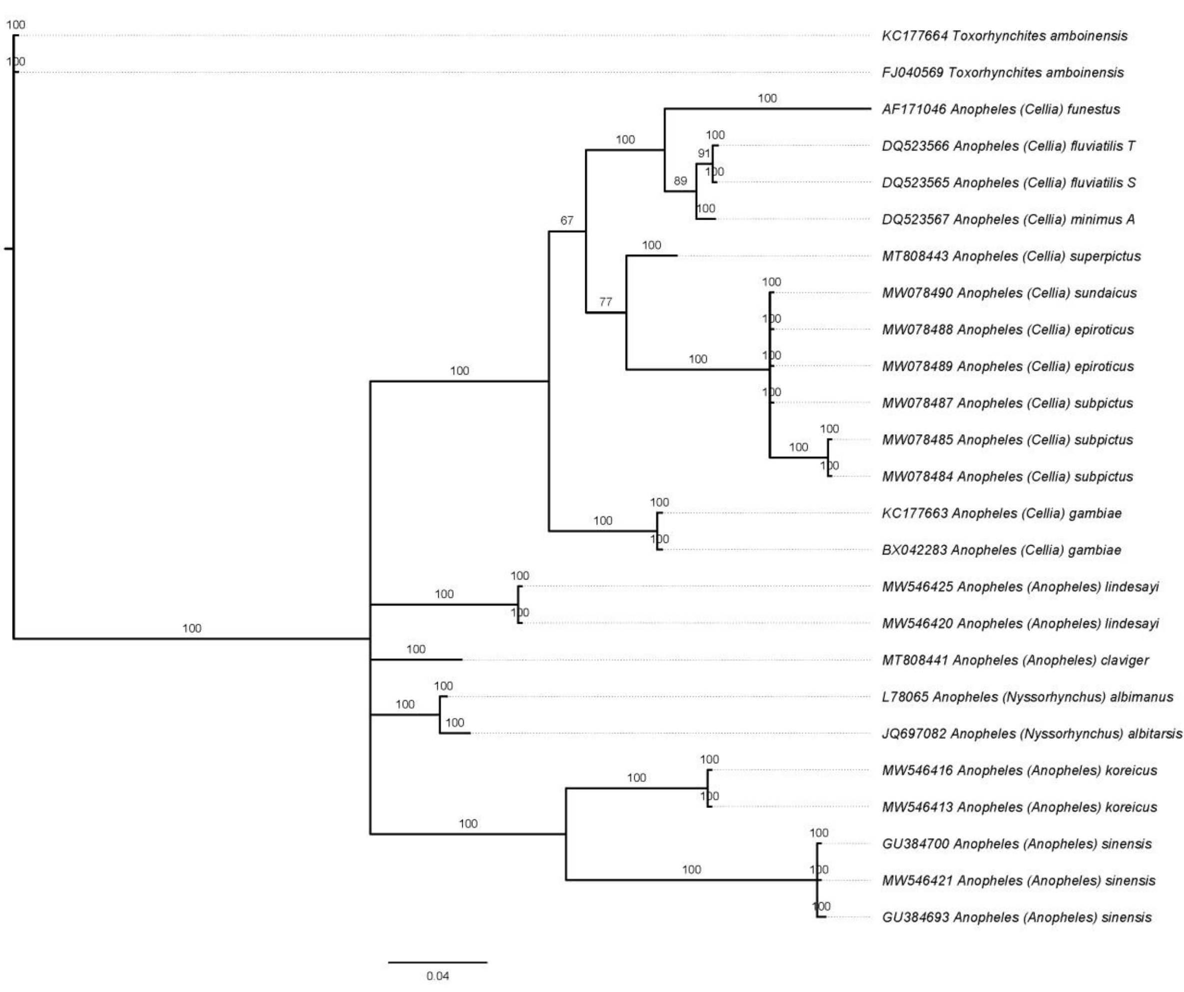


5.5.17 *ANOPHELES*, 28S, BAYESIAN ANALYSIS



5.5.18 *ANOPHELES*, 28S, MAXIMUM LIKELIHOOD ANALYSIS