Phylogeny of anopheline (Diptera: Culicidae) species in southern Africa, based on nuclear and mitochondrial genes

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ABSTRACT**:** A phylogeny of anthropophilic and zoophilic anopheline mosquito species was constructed, using the nuclear internal transcribed spacer 2 (ITS2) and mitochondrial cytochrome oxidase subunit I (COI) genes. The ITS2 alignment, typically difficult due to its noncoding nature and large size variations, was aided by using predicted secondary structure, making this phylogenetically useful gene more amenable to investigation. This phylogeny is unique in explicitly including zoophilic, non-vector anopheline species in order to illustrate their relationships to malaria vectors. Two new, cryptic species, *Anopheles funestus*-like and *Anopheles rivulorum*-like, were found to be present in Zambia for the first time. Sequences from the D3 region of the 28S rDNA suggest that the Zambian *An. funestus*-like may be a hybrid or geographical variant of *An. funestus*-like, previously reported in Malawi. This is the first report of *An. rivulorum*-like sympatric with *An. rivulorum* (Leeson), suggesting that these are separate species rather than geographic variants. ***Journal of Vector Ecology* 40 (1): 16-27. 2015.**

Keyword Index: Anopheles, phylogeny, ITS2, COI, Zambia.

INTRODUCTION

The genus *Anopheles* contains over 400 species (Harbach 2004), of which 30-40 are vectors for human malaria. The *Anopheles* genus is subdivided into six subgenera, *Anopheles*, *Cellia, Kerteszia*, *Lophopodomyia*, *Nyssorhynchus*, and *Stethomyia*. All *Anopheles* in sub-Saharan Africa are grouped in *Anopheles* and *Cellia* (Gillies and DeMeillon 1968). The Anopheles subgenus is further divided into the Angusticorn and Laticorn Series. The Cellia subgenus is divided into the Cellia, Myzomyia, Neocellia, Pyretophorus, Neomyzomyia, and Paramyzomyia Series.

Most phylogenetic work on the *Anopheles* genus has either focused on higher-level taxonomy (Krzywinski et al. 2001, Sallum et al. 2002, Sallum et al. 2000) or on elucidating relationships between major malaria vectors and their closely related taxa (Anthony et al. 1999, Garros et al. 2004, Marshall et al. 2005), see (Harbach 2004) for review. In contrast, this study encompasses primary, secondary, and nonvector anophelines present in Zambia and southern Africa.

For the purposes of this study, we included sympatric anopheline species present in southern Zambia (Table 1) (*Anopheles gambiae* Giles, *An. arabiensis* Patton, *An. quadriannulatus* Theobald species A, *An. pharoensis* Theobald, *An. squamosus* Theobald, *An. rufipes* Gough, *An. maculipalpis* Giles, *An. pretoriensis* Theobald, *An. coustani* sensu lato Laveran, *An. theileri* Edwards, *Anopheles rivulorum* Leeson, *An. leesoni* Evans, *An. funestus* sensu strictu Giles, *An. rivulorum*-like, *An. funestus*-like), as well as species from South Africa (*Anopheles vaneedeni* Gillies & Coetzee, *Anopheles parensis* Gillies) that are closely related but with limited range. *An. minimus* Theobald species A from Thailand was included due to its close relatedness to *An. leesoni*. ITS2 sequences were downloaded from GenBank for *An. minimus* Theobald species C, *An. rivuloum*-like from Burkina Faso, and *An. funestus*-like from Malawi.

Anopheles gambiae, An. arabiensis, and *An. quadriannulatus* belong to the *An. gambiae* s.l. complex (Pyretophorus Series) and all have a wide distribution throughout Africa, with *An. gambiae* restricted to wetter areas, while *An. arabiensis* is more tolerant to drought (Gillies and DeMeillon 1968). *An. gambiae* is highly anthropophilic and one of the most important malaria vectors in sub-Saharan Africa (Gillies and DeMeillon 1968, White et al. 1972). *An. arabiensis* can vary in anthropophilicity, depending on locale, and has been shown to be primarily anthropophilic in southern Zambia (Fornadel et al. 2010a, Kent et al. 2007) and is the primary malaria vector in that region. *An. quadriannulatus,* although closely related to these two highly efficient vectors, is almost entirely zoophilic and therefore does not vector malaria. *An. quadriannulatus* is now known to be composed of two species, A and B. In Macha, only *An. quadriannulatus* A has been collected, therefore *An. quadriannulatus* B was not included.

Anopheles pharoensis and *An. squamosus* (Cellia Series) have mainly been ignored as vectors due to the fact that they are considered zoophilic. However, dissections of *An. squamosus* have yielded *Plasmodium* sporozoites in Tanzania (Gillies 1964) and Zimbabwe (Gillies and DeMeillon 1968), and they have been shown to bite humans in Zambia (Fornadel et al. 2010b). *An. pharoensis* has been shown to bite humans in high numbers and have been implicated as a major vector in Egypt (Barber and Rice 1937) and some areas of Cameroon (Antonio-Nkondjio et al. 2006), and as a secondary vector in Senegal (Dia et al. 2008). Its variation in vector status and behavior, as well as chromosomal inversion studies, suggests that *An. pharoensis* may be a species complex (Miles et al. 1983).

Anopheles rufipes, *An. maculipalpis,* and *An. pretoriensis*

(Neocellia Series), are almost entirely zoophilic. Because *An. rufipes* occasionally enters households and bites people, and due to a small number of positive sporozoite dissections, it has been considered a secondary vector (Gillies and DeMeillon 1968). *Anopheles coustani* s.l. (*Anopheles* subgenus, Laticorn series) is outside the Cellia subgenus and is therefore used here as an outgroup. It has highly variable behavior, with few collected in human landing catches in Cameroon (Antonio-Nkondjio et al. 2006), Kenya (Mbogo et al. 1995), and Senegal (Dia et al. 2008), but increased anthropophily reported in Ethiopia (Taye et al. 2006), South Africa (Coetzee 1983), Mozambique (Mendis et al. 2000), and Zambia (Fornadel et al. 2010b). Additionally, specimens infected with *Plasmodium malariae* have been found in Cameroon (Antonio-Nkondjio et al. 2006). Like *An. pharoensis*, its variability in behavior prompted studies which indicated that *An. coustani* s.l. includes at least two species, *An. coustani* sensu strictu and *An. crypticus* (Coetzee 1994).

The Funestus Group, in the Myzomyia series, includes the Rivuloum Subgroup (*An. rivulorum*, *An. rivulorum*-like, *An. brucei*, *An. fuscivenosus*, all Afrotropical), the Minimus Subgroup (*An. minimus* A, C, and E, *An. leesoni, An. fluviatilis, An. flavirostris*, all Asian except *An. leesoni*), and the Funestus Subgroup (*An. funestus* s.s., *Anopheles vaneedeni*, *Anopheles parensis*, and *An. aruni,* all Afrotropical) (Garros et al. 2005, Harbach 2004). These species are morphologically similar as adults, and adults in the Funestus Subgroup are indistinguishable (Gillies and DeMeillon 1968), but the most common species are easily distinguished by PCR diagnostic (Koekemoer et al. 2002).

Despite their morphological similarity, mosquitoes in the Funestus Group have variable host preferences and vector competence. The anthropophilic *An. funestus* s.s. is a highly competent malaria vector, rivaling *An. gambiae* ss. as a major vector in Africa. *An. rivulorum* (Wilkes et al. 1996) and *An. vaneedeni* (De Meillon et al. 1977) are capable of *Plasmodium* transmission but are primarily zoophilic and therefore considered secondary vectors, and all other members of the complex are zoophilic (Cohuet et al. 2003). *An. minimus* s.l. is anthropophilic and one of the primary malaria vectors in Southeast Asia (Harrison 1980), but the related *An. leesoni* is primarily zoophilic and a non-vector (Gillies and DeMeillon 1968).

Anopheles theileri is in the Wellcomei Group, Myzomyia Series, and is almost entirely zoophilic (Gillies and DeMeillon 1968). *An. longipalpis*, also in the Myzomyia Series, includes two cryptic species, Type A and Type C (Koekemoer et al. 2009), and Type C was used in this study. *Anopheles longipalpis* is almost entirely zoophilic (Gillies and DeMeillon 1968) but can be highly endophilic, making it easily mistaken for the vector *An. funestus* s.s. (Kent et al. 2007). Additionally, two cryptic species, *Anopheles rivulorum*-like and *Anopheles funestus*-like, have been discovered based upon divergent ITS2 sequences (Cohuet et al. 2003, Spillings et al. 2009).

Specimens of *Anopheles rivulorum*-like have only been found in West Africa, particularly Cameroon (Cohuet et al. 2003) and Burkina Faso (Hackett et al. 2000), while *An. rivulorum* is confined to southern Africa. The vector status of *An. rivulorum*-like is unknown. Prior to this, there have been no reports of these two species in the same location, however, here we document that they are sympatric in southern Zambia. *Anopheles funestus*-like was recently identified in Malawi, where it is sympatric with *An. funestus* s.s., but experiments have definitively shown the two species to be separate (Spillings et al. 2009). In Macha, *An. funestus* s.s. was collected prior to a drought in 2004-2005 (Kent et al. 2007), after which no specimens were collected for several years and it was presumed that the species was locally extinct. In 2008-2009, several specimens of both *An. funestus* s.s. and *An. funestus*-like were collected.

As part of ongoing entomological research in southern Zambia, our aim was to construct a phylogeny of anopheline species in southern Africa, including previously overlooked zoophilic and non-vector species. By including all species present in this geographic area, we hope to shed light on the evolution of characteristics such as anthropophilicity, endophily, adaption to human-altered environments, and vector competence for malaria.

MATERIALS AND METHODS

Study site and sample collection

The Johns Hopkins Malaria Research Institute field station in Macha, Zambia is located in the southern province at 16.39292° S, 26.79061° E at an elevation of approximately 1,100 m above sea level. The habitat around the field station, the Malaria Institute at Macha (MIAM), is Miombo woodland. Mosquitoes were collected by CDC light trap (Beier 2002, Fornadel et al. 2010a, Sudia and Chamberlain 1988), human landing collection (Service 1976), and by cattle-baited trap (Fornadel et al. 2010b) in four village areas within 10 km of the field station. All samples were identified morphologically (Gillies and Coetzee 1987) and packaged on silica gel for transport to Johns Hopkins. Samples from the *An. gambiae* complex were further molecularly identified by the diagnostic developed by Scott and others (Scott et al. 1993), and samples from the *An. funestus* group were identified by the diagnostic developed by Koekomer and others (Koekemoer et al. 2002). Blood meal host species were identified for samples of *An. funestus* s.s. and *An. rivulorum*-like by PCR (Fornadel and Norris 2008, Kent and Norris 2005). Samples of mosquito species not native to southern Zambia were obtained as follows: *An. gambiae* from the Keele strain maintained at the Johns Hopkins School of Public Health insectary, *An. minimus* A collected in Thailand, *An. funestus*, *An. leesoni*, *An. rivulorum,* and *An. vaneedeni* from South Africa were provided by Maureen Coetzee and Lizette Koekemoer. The ITS2 sequences of *An. minimus* C (accession no. AF 230462.1), *An. funestus-like* from Malawi (GenBank accession no. FJ438963), and *An. rivulorum-like* from Cameroon and Burkina Faso (GenBank accession no. AF210725.1) were downloaded from GenBank.

Gene targets used in phylogenetic analyses

The cytochrome c oxidase I subunit (COI) is a mitochondrial protein-coding gene that has often been used for anopheline phylogeny construction (Marshall et al. 2005, Mohanty et al. 2009, Sallum et al. 2002) due to its phylogenetic information content, reliable PCR amplification due to high copy number, and ease of alignment due to evolutionary constraints imposed by the triplet code and necessity of coding for a functional enzyme. The internal transcribed spacer 2 is a nuclear, non-coding gene that separates the 5.8S and 28S large subunit RNA genes on the ribosomal RNA precursor transcript. The rRNA genes are arrayed

Table 1. Behavioral and vectorial characteristics of species in this study. Major vectors are marked in bold and "potential secondary" indicates that the species has been shown to be capable of transmitting *Plasmodium* but is unlikely to be a vector due to mainly zoophilic feeding behavior.

Species	anthropophilic vs zoophilic	endophilic vs. exophilic	vector status
An. arabiensis	variable	variable	**major vector**
An. coustani	variable, mainly zoophilic	mainly exophilic	potential secondary
An. funestus	highly anthropophilic	endophilic	**major vector**
An. funestus-like	potentially zoophilic	unknown	unknown
An. gambiae s.s.	highly anthropophilic	endophilic	**major vector**
An. leesoni	zoophilic	exophilic	nonvector
An. longipalpis	zoophilic	frequently endophilic	nonvector
An. maculipalpis	zoophilic	exophilic	nonvector
An. minimus	highly anthropophilic	endophilic	**major vector**
An. parensis	zoophilic	exophilic	nonvector
An. pharoensis	mainly zoophilic	mainly exophilic	potential secondary
An. pretoriensis	zoophilic	exophilic	nonvector
An. quadriannulatus	zoophilic	exophilic	nonvector
An. rivulorum	zoophilic	exophilic	potential secondary
An. rivulorum-like	potentially variable	unknown	unknown
An. rufipes	zoophilic	exo-	potential secondary
An. squamosus	mainly zoophilic	mainly exophilic	potential secondary
An. theileri	zoophilic	exophilic	nonvector
An. vaneedeni	zoophilic	exophilic	potential secondary

in tandem repeats of several thousand, and the flanking 5.8S and 28S genes provide highly conserved primer binding sites, facilitating PCR amplification. Despite the fact that there are thousands of copies of the ribosomal RNA cistron, the sequences are homogenized by concerted evolution, such that high quality gene sequences can be obtained (Coleman 2003, Hershkovitz 1999). Unlike protein-coding genes, the ITS2 is not constrained by amino acid coding and is therefore more variable than most coding genes used for phylogeny reconstruction, giving greater resolution (Coleman 2003, Schultz et al. 2005). A major drawback is the high sequence variation and differences in length that can make alignment of sequences from taxonomic levels above species difficult or impossible. However, during rRNA maturation, the ITS2 folds into a secondary structure necessary for ribosomal cistron processing (Venema and Tollervey 1999). This secondary structure is greatly conserved among eukaryotes (Coleman and Vacquier 2002, Mai and Coleman 1997, Michot et al. 1999, Schultz et al. 2005) and can be used to aid primary sequence alignment. This conserved secondary structure, which can be used to identify the proper secondary structure from predicted models, consists of four helix loop regions, of which helix II is highly conserved and helix III is the longest, a pyrimidine mismatch in the basal 7 nucleotide pairs of helix II, and a conserved UGGU sequence at the 5' end of helix III (Schultz et al. 2005). The ITS2 has been widely used for phylogeny studies (Coleman 2003), especially in plants (Hershkovitz 1999), and has more recently been extended to insects (Wiemers et al. 2009, Young and Coleman 2004), but despite investigations into mosquito ITS2 sequence and structure (Paskewitz et al. 1993, Wesson et al. 1992) and use of ITS2 sequences for mosquito species identification (Cohuet et al. 2003, Garros et al. 2004, Koekemoer et al. 2002, Spillings et al. 2009), its phylogenetic use for *Anopheles* has been limited to the subspecies level (Dassanayake et al. 2008).

DNA extraction and sequencing

DNA was extracted from dried mosquito samples using a DNeasy® Blood and Tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The variable internal transcribed spacer 2 region (ITS2) was amplified with primers from the flanking 5.8S and 28S genes, ITS2A (5'-TGT GAA CTG CAG GAC ACA T-3') and ITS2B (5'-ACC CCC TGA ATT TAA GCA TA-3') (Koekemoer et al. 2002). Because sequencing reactions with ITS2A frequently failed, some samples were amplified and sequenced using the novel primer ITS2B1 (5'-GTC CCT ACG TGC TGA GCT TC-3'). This primer binds further downstream in the 28S gene, such that the 3' portions of products immediately upstream of the ITS2B binding site could be sequenced. Each 25 µl reaction contained 1X PCR buffer, 200µM each dNTP, 30 pmol each primer, two units Taq polymerase, and 1.0 µl DNA template. Products were amplified in a thermocycler (MJ Research, Watertown, MA, USA) using the following conditions: 2 min initial denaturation at 94° C, 40 cycles of 30 s at 94° C, 30 s at 50° C, and 40 at 72° C, and a 10 min final extension at 72° C. PCR product size ranged from 480 bp to 847 bp. *An. squamosus* and

An. pharoensis (both Cellia Series) repeatedly failed to produce an ITS2 PCR product despite varied PCR conditions and the creation of new primers, and were therefore not included in the ITS2 tree.

An 877 bp fragment of the cytochrome oxidase subunit I (COI) gene was amplified with the primers COI (5'-TTG ATT TTT TGG TCA TCC AGA AGT-3') and Cul-Rev (5'-TAG AGC TTA AAT TCA TTG CAC TAA TC-3') (Oshaghi et al. 2006). PCR reaction mixtures were identical to the ITS2 reaction, with the following thermocycler conditions: 1 min initial denaturation at 94° C, 32 cycles of 1 min at 94° C, 1 min at 55° C, 2 min at 72° C, and a 7 min final extension at 72° C.

Domain 3 of the 28S rDNA (D3) was sequenced from samples of *An. funestus*-like from Zambia for comparison to published sequences of *An. funestus* s.s. and *An. funestus*-like from Malawi (GenBank accession no. DQ407757.1 and FJ843022, respectively) (Spillings et al. 2009). The product was amplified with the primers D3A (5'-GAC CCG TCT TGA AAC ACG GA-3') and D3B (5'-TCG GAA GGA ACC AGC TAC TA-3'). Each 25 µl reaction contained 1X PCR buffer, 200 µM of each dNTP, 25 pmol of each primer, 2 U *Taq* polymerase, and 1 µl DNA template and was amplified using the following conditions: 3 min initial denaturation at 94° C, followed by 30 cycles of 94° C denaturation for 30 s, 63° C annealing for 40 s, 72° C extension for 40 s, and a 72° C final extension for 10 min.

Five µl of each PCR product was subjected to electrophoresis on a 2% agarose gel, stained with ethidium bromide, and visualized under UV illumination. The remainder of each successful PCR reaction was purified using a Qiaquick PCR prep kit (Qiagen, Valenca, CA). Products were sequenced in both directions using dye terminator chemistry on a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA) at the Johns Hopkins University School of Medicine Sequencing and Synthesis Facility. Accession numbers for each sequence are given in Table 2.

ITS2 PCR products from South African samples of *An. leesoni*, *An. funestus*, and *An. rivulorum* and COI PCR products from *An. leesoni* and *An. funestus* were 100% identical to their Zambian counterparts. They were therefore dropped from the alignment.

Alignment and phylogeny construction

The Clustal function in MEGA 4.0 (Tamura et al. 2007) was used to align the COI sequences and to trim the ends of sequences, and MEGA 4.0 was used to build phylogenies. For the Neighbor-Joining (NJ) tree, evolutionary distances were computed using the Maximum Composite Likelihood evolution method. The 1st+2nd+3rd+non-coding codon positions were used. Gaps and missing data were completely deleted from the dataset, leaving a total of 831 positions. The Maximum Parsimony (MP) tree was obtained using the Close-Neighbor-Interchange algorithm with a search level of 3, in which the initial trees were obtained with the random addition of sequences (ten replicates). The 1st+2nd+3rd+non-coding codon positions were used. Trees were rooted using *An. coustani* s.l. as an outgroup. Bootstrap tests with 1,000 replicates were performed for both trees. NJ and MP trees using translated COI protein sequences were constructed similarly but were poorly supported at most nodes (bootstrap value <50) and therefore not shown.

The 5.8S and 28S ends of the ITS2 were determined using the HMM Annotation tool on the ITS2 database website (Keller et al. 2009) at http://its2.bioapps.biozentrum.uni-wuerzburg.de, and sequences were trimmed to this length to remove the highly conserved fragments of the 5.8S and 28S rRNA genes that border the ITS2. Secondary structures were determined by custom homology modeling using the ITS2 database (Wolf et al. 2005) (http://its2.bioapps.biozentrum.uni-wuerzburg.de/cgi-bin/index.pl?custom). *Anopheles superpictus* (GenBank accession number EU482198) was used as the template. For sequences that did not have significant similarity to *An. superpictus,* the *An. pretoriensis* secondary structure result was used as a template. ITS2 sequences were aligned using 4SALE (Seibel et al. 2006), which takes secondary structure into account during alignment. Phylogenetic trees were then constructed using the same NJ and MP methods described for COI, with the difference that gaps and missing data were deleted in only pairwise comparisons.

Because the ITS2 sequences of *An. funestus-like* from Malawi (GenBank accession no. FJ438963) and *An. rivulorum-like* from Cameroon and Burkina Faso (GenBank accession no. AF210725.1) are shorter than the sequences used in our phylogeny, separate alignments of these sequences with their Zambian counterparts were constructed in MEGA 4.0. Pairwise genetic distances were calculated as number of base substitutions per site, using the Maximum Composite Likelihood method and with gaps and missing data deleted only for pairwise comparisons. Additionally, the *An. funestus-like* Zambia D3 partial sequence was aligned with published *An. funestus-like* and *An. funestus* s.s. sequences using MEGA 4.0.

RESULTS

COI phylogenies

In the COI alignment, there were 831 positions in the dataset, 188 of which were parsimony informative. For the NJ tree, the optimum tree (with a branch length of 0.75231908) is shown (Figure 1) with bootstrap values at each node. This tree maintained all series as monophyletic groups, placing Cellia closest to Pyretophorus, and Neocellia as the outermost group. The Rivulorum and Minimus Subgroups within Myzomyia were monophyletic, *An. theileri* was placed within the Funestus Group, and *An. longipalpis* was placed within the Funestus Subgroup.

The Maximum Parsimony method found 12 most parsimonious trees, with a length of 639, one of which is shown (Figure 2). These 12 trees differed only in the internal arrangement of the clade containing *An. vaneedeni*, *An. funestus* s.s., *An. funestus*-like, *An. parensis,* and *An. longipalpis*. Six of the 12 trees included *An. longipalpis* within the Funestus Subgroup, while six put it outside the clade. This tree did not preserve the Cellia and Neocellia series but still put *An. pharoensis* closest to the *An. gambiae* complex. However, most higher-level nodes were poorly supported in both COI trees.

ITS2 phylogenies

In the ITS2 alignment, there were 658 positions in the final dataset, of which 277 of which were parsimony informative. For the Neighbor-Joining method, the optimum tree (with a branch length of 5.06003986) is shown (Figure 3) with bootstrap values at each node. The Maximum Parsimony method found three most parsimonious trees, with a length of 902. These three trees

Table 2. Gene name, species, location collected, and GenBank accession numbers of samples used in phylogeny construction.

Gene	Species	Location	Accession number
ITS2	*An. arabiensis*	Zambia	JN994133
ITS2	*An. coustani*	Zambia	JN994134
ITS2	*An. funestus*	Zambia	JN994135
ITS2	*An. funestus*	South Africa	JN994136
ITS2	*An. funestus-like*	Zambia	JN994137
ITS2	*An. gambiae*	Keele strain colony	JN994138
ITS2	*An. leesoni*	Zambia	JN994139
ITS2	*An. longipalpis* upper band	Zambia	JN994140
ITS2	*An. longipalpis* lower band	Zambia	JN994141
ITS2	*An. maculipalpis*	Zambia	JN994142
ITS2	*An. minimus C*	Thailand	JN994143
ITS2	*An. parensis*	Zambia	JN994144
ITS2	*An. pretoriensis*	Zambia	JN994145
ITS2	*An. quadriannulatus*	Zambia	JN994146
ITS2	*An. rivulorum-like*	Zambia	JN994147
ITS2	*An. rivulorum*	South Africa	JN994148
ITS2	*An. rivulorum*	Zambia	JN994149
ITS2	*An. rufipes*	Zambia	JN994150
ITS2	*An. theileri*	Zambia	JN994151
ITS2	*An. vaneedeni*	South Africa	JN994152
COI	*An. arabiensis*	Zambia	JN994153
COI	*An. coustani*	Zambia	JN994154
COI	*An. funestus*	Zambia	JN994155
COI	*An. funestus*-like	Zambia	JN994156
COI	*An. gambiae* s.s.	Keele strain colony	JN994157
COI	*An. leesoni*	Zambia	JN994158
COI	*An. longipalpis*	Zambia	JN994159
COI	*An. maculipalpis*	Zambia	JN994160
COI	*An. minimus C*	Thailand	JN994161
COI	*An. parensis*	Zambia	JN994162
COI	*An. pharoensis*	Zambia	JN994163
COI	*An. pretoriensis*	Zambia	JN994164
COI	*An. quadriannulatus*	Zambia	JN994165
COI	*An. rivulorum-like*	Zambia	JN994166
COI	*An. rivulorum*	South Africa	JN994167
COI	*An. rivulorum*	Zambia	JN994168
COI	*An. rufipes*	Zambia	JN994169
COI	*An. squamosus*	Zambia	JN994170
COI	*An. theileri*	Zambia	JN994171
COI	*An. vaneedeni*	South Africa	JN994172

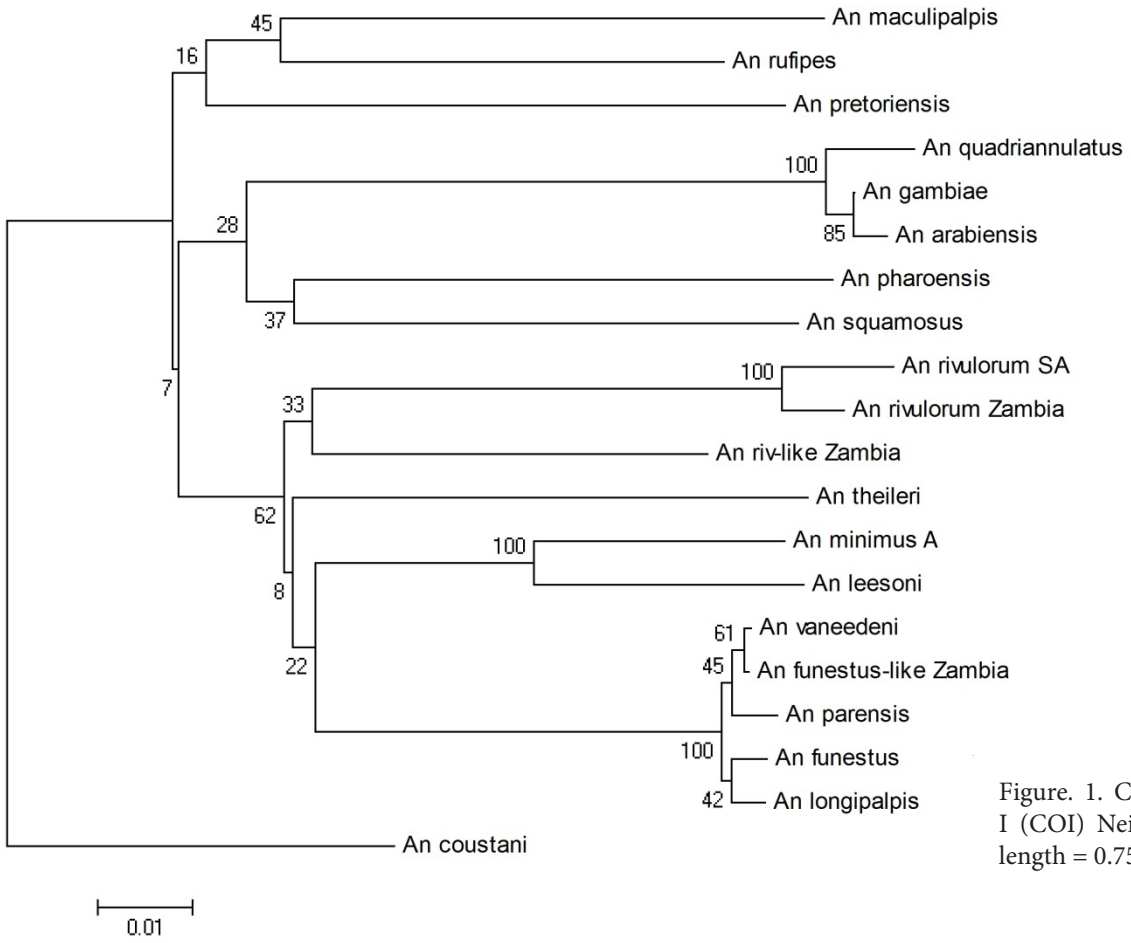

Figure. 1. Cytochrome oxidase subunit I (COI) Neighbor-Joining tree, branch length = 0.75231908.

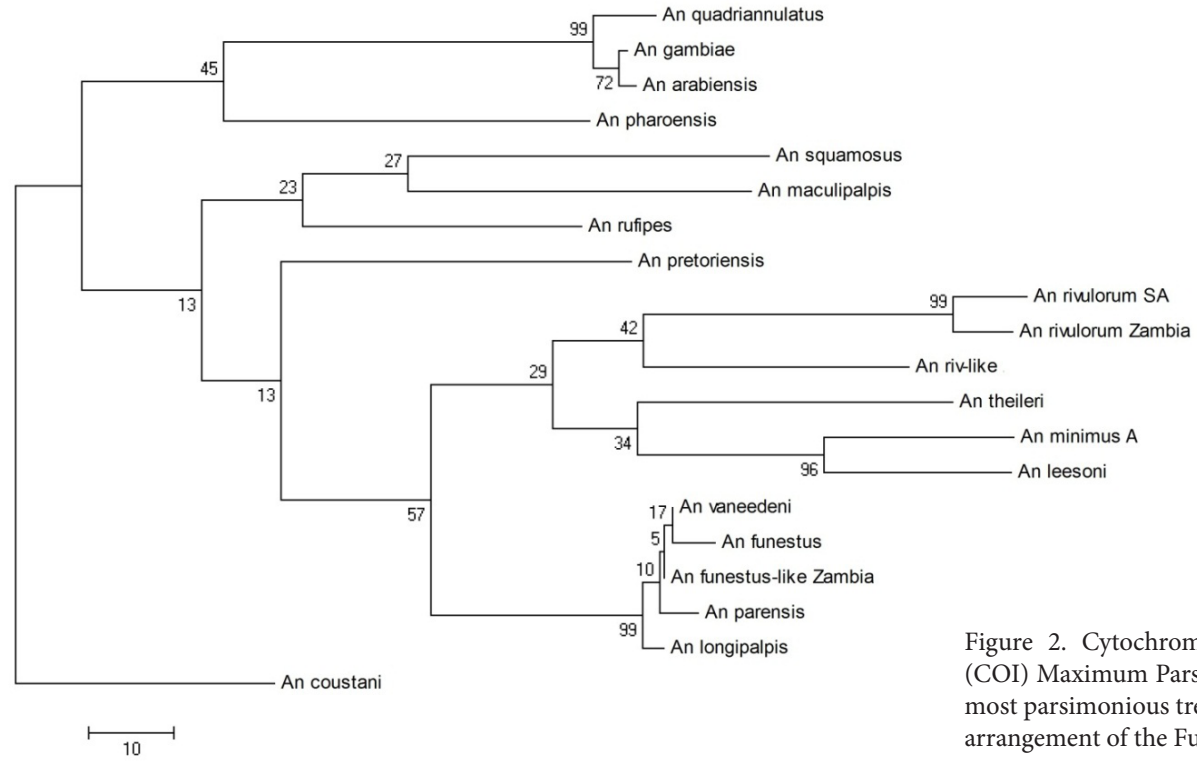

Figure 2. Cytochrome oxidase subunit I (COI) Maximum Parsimony tree, one of 12 most parsimonious trees that differed in the arrangement of the Funestus Group clade.

differed only in the rearrangement of the *An. gambiae* complex clade. One of these trees is shown (Figure 4). The MP tree was in best agreement with the current classification of the *Anopheles* genus (Harbach 2004): the Neocellia Series (*An. maculipalpis*, *An. pretoriensis*, and *An. rufipes*) and Pyretophorus Series (*An. gambiae* complex) are monophyletic. However, the resulting phylogeny has the Myzomyia Series as paraphyletic, with *An. minimus, An. leesoni, An. theileri, An. rivulorum*, and *An. rivulorum*-like making up one clade, and *An. vaneedeni, An. parensis, An. longipalpis, An. funestus* and *An. funestus*-like making up the other. The Minimus and Rivulorum Subgroups are each monophyletic, and the Funestus Subgroup groups together but includes *An. longipalpis*. The ITS2 NJ tree is arranged nearly identically to the MP tree, with the exception that the Minimus and Rivulorum Subgroups are separate clades.

During the 2008-2009 rainy season, two specimens of *An. funestus* s.s., four specimens of *An. funestus*-like, one specimen of *An. rivulorum*, and 16 specimens of *An. rivulorum*-like were collected from nearby localities in southern Zambia. All were collected in CDC light traps inside houses, except for two *An. rivulorum*-like from cattle-baited traps and one from an indoor human landing catch. One *An. funestus* s.s. was blooded (human), as well as two *An. funestus*-like (one human, one goat), and five *An. rivulorum*-like (one human, one human+cow, one human+goat, two cows) (Table 3). Additionally, one archived *An. funestus*-like sample collected by pyrethroid spray catch in 2006 was identified by ITS2 sequencing and contained cow blood. All other specimens were unfed. ITS2 sequences were identical for all samples within each species.

Although there was no ITS2 divergence within *An. rivulorum* and *An. funestus* s.s. species from Zambia and South Africa, the sequences of *An. rivulorum*-like and *An. funestus*-like from Zambia were divergent from their counterparts in Burkina Faso and Malawi, respectively. Out of 449 total basepairs, *An. rivulorum*-like Zambia had 15 basepair substitutions and a one bp insertion when compared to *An. rivulorum*-like Burkina Faso (Table 4). Of 740 total basepairs, *An. funestus*-like Zambia had eight substitutions and a four bp insertion when compared to *An. funestus*-like Malawi (Table 5). Interestingly, the D3 sequence of *An. funestus*-like Zambia was closest to that of *An. funestus* s.s., with only a single one bp deletion, vs a one bp insertion and two substitutions when compared to *An. funestus*-like Malawi (Figure 5).

DISCUSSION

A current theory suggests that the *Anopheles* ancestral phenotype is resistance to malaria (Riehle et al. 2006, Rios-Vasquez et al. 2013). Using this assumption, previous work has shown independent evolution of anthropophily and *Plasmodium* vectorial capacity has occurred multiple times within major African vector species (Marshall et al. 2005, Kamali et al. 2012). By including zoophilic species, our phylogeny suggests that anthropophilicity and capacity to vector *Plasmodium* evolved independently at least three times within the subgenus Cellia, with *An. gambiae* s.s. and *An. arabiensis*; *An. minimus* A and C; and *An. funestus* s.s. clustering more closely with zoophilic species than with each other. Intermediate levels of anthropophilicity, and minor vector status, have evolved at least twice, in the clade containing *An. pharoensis* and *An. squamosus*, and in *An. rivulorum*. Alternatively, if the ability to transmit *Plasmodium* is the ancestral phenotype, this trait was lost at least three times.

Historically, the status of *An. longipalpis* has been disputed (Koekemoer et al. 2009). Despite variation in the arrangement of the Funestus Subgroup, all the trees placed *An. longipalpis* C within this clade, with the exception of half the COI MP trees that placed *An. longipalpis* C immediately outside the clade. This agrees with previous work (Koekemoer et al. 2009) showing that *An. longipalpis* C is more closely related to the Funestus Subgroup than to the Minimus Subgroup. Additionally, all trees placed *An. theileri*, a member of the Wellcomei Group, within the Funestus Group, indicating that either the Funestus Group is not monophyletic, or that the Wellcomei Group should be included within it.

For distal nodes, the ITS2 trees appeared to be more robust than the COI trees. The use of secondary structure greatly assisted in properly aligning sequences, making it possible to use this gene. The ITS2 contained more parsimony informative sites by length (277 of 658 bp vs 188 of 831 bp for COI) and provided trees with higher bootstrap values at most nodes. Unlike the COI NJ and MP trees, which were not highly similar, the ITS2 trees had nearly identical topology, indicating that the ITS2 was less sensitive to which method was used. However, as shown by bootstrap values at higher-level nodes, the more variable ITS2 was less accurate in determining basal tree topology. Both trees inaccurately placed *An. gambiae* and *An. minimus* groups together, despite morphological evidence that *An. minimus* and *An. funestus* are sister taxa (Marshall et al. 2005, Garros et al. 2005). Another limitation was the inability to PCR amplify the ITS2 from species in the Cellia Series.

An additional consideration is whether these trees may be affected by introgression. In the *An. gambiae* group, autosomal genes and inversions have introgressed between sibling species, complicating the construction of phylogenetic trees (Besansky et al. 2003, Besansky et al. 1994). However, the X chromosome, particularly the pericentromeric region which contains the rDNA and ITS2 locus, is more divergent and less susceptible to introgression (Besansky et al. 2003).

This is the first report in southern Africa of *An. rivulorum*-like, previously limited to West Africa (Cohuet et al. 2003). *An. rivulorum* and *An. rivulorum*-like are sympatric in Zambia and there is no evidence of hybridization, which lends more weight to the theory that *An. rivulorum*-like is a separate species. Specimens of *An. rivulorum*-like from Zambia all had identical ITS2 sequences but were divergent from ITS2 sequences from West Africa. However, they were clearly more closely related to *An. rivulorum*-like than to *An. rivulorum* and may simply be geographic variants. In Zambia, *An. rivulorum*-like appears to be at least somewhat anthropophilic, as they were collected in indoor CDC traps and human landing catches, and over half the blooded samples contained either human or mixed human/animal blood meals. However, it is unknown how many were collected in cattle-baited traps as our lab does not routinely conduct molecular species diagnostic assays to distinguish *An. rivulorum*-like from the very common *An. longipalpis* collected in cattle-baited traps. Because the closely related *An. rivulorum* is known to be a

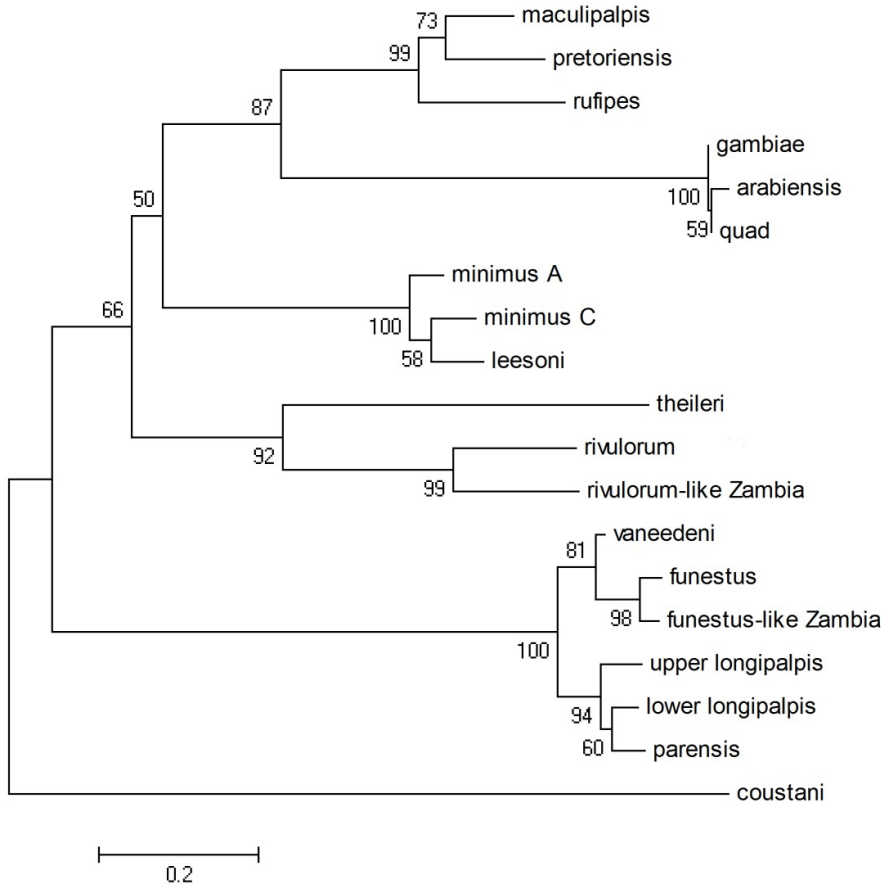

Figure 3. Internal transcribed spacer 2 (ITS2) Neighbor-Joining tree, branch length = 5.06003986.

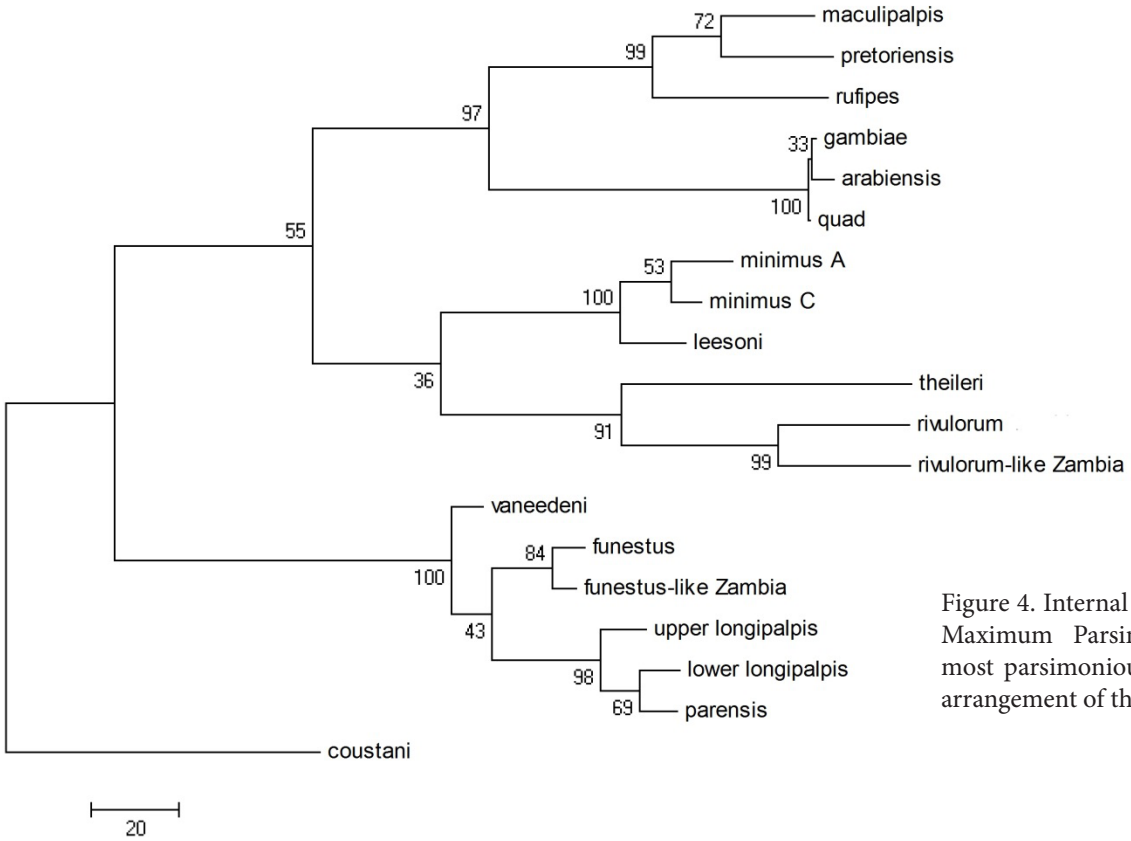

Figure 4. Internal transcribed spacer 2 (ITS2) Maximum Parsimony tree, one of three most parsimonious trees that differed in the arrangement of the *An. gambiae* clade.

Table 3. *An. funestus* s.s., *An. funestus*-like, *An. rivulorum,* and *An. rivulorum*-like samples collected in Macha during the 2008-2009 rainy season. Locations in Universal Transverse Mercator grid 35L: Namwalinda (X 487921, Y8193087), Chidakwa (X 477263, Y8184855), Lupata (X475497, Y8188941), and regional collections (X495271, Y8192700).

	# collected	blood meals	location
An. funestus s.s.	2	1 human	Namwalinda
An. funestus-like	5	1 cow	Namwalinda and Chidakwa
An. rivulorum	3	--	Namwalinda
An. rivulorum-like	20	1 human, 1 human+cow, 1 human+goat, 3 cows	Namwalinda, Lupata, and Chidakwa

Table 4. Table of pairwise differences for *An. rivulorum* and *An. rivulorum*-like ITS2 sequences. Number of base substitutions per site; analysis conducted using Maximum Composite Likelihood method. Gaps and missing data were deleted only in pairwise comparisons.

	rivulorum-like Burkina Faso	*rivulorum*-like Zambia	*rivulorum*
rivulorum-like Burkina Faso	---		
rivulorum-like Zambia	0.035	---	
rivulorum	0.165	0.170	---

Table 5. Table of pairwise differences for *An. funestus* s.s. and *An. funestus*-like ITS2 sequences. Number of base substitutions per site; analysis conducted using Maximum Composite Likelihood method. Gaps and missing data were deleted only in pairwise comparisons.

	funestus-like Malawi	*funestus*-like Zambia	*funestus* s.s.
funestus-like Malawi	---		
funestus-like Zambia	0.010	---	
funestus s.s.	0.037	0.036	---

potential secondary vector, it remains possible that *An. rivulorum*-like could vector malaria.

Additionally, this is the first report of *An. funestus*-like outside of Malawi. Like *An. rivulorum*-like, all Zambian ITS2 and D3 sequences were identical. However, it is unclear if this is an *An. funestus* s.s. x *An. funestus*-like hybrid, or an *An. funestus*-like geographic variant. The ITS2 sequence is more closely related to that of *An. funestus*-like, but the D3 sequence is clearly closer to *An. funestus* s.s. Differences as small as 2-3 bp in the D3 region have been shown to differentiate between species (Singh et al. 2004, Spillings et al. 2009), indicating that Zambian *An. funestus*-like may be a separate species. In Zambia, *An. funestus*-like enters houses and at least occasionally feeds on humans. Because *An. funestus* s.s. is such an efficient malaria vector, it will be important to monitor *An. funestus*-like to determine if it is also a malaria vector.

This phylogeny, which includes all *Anopheles* species present in southern Zambia, as well as many from southern Africa, clarifies the relationships between the anthropophilic malaria vectors *An. gambiae* s.s., *An. arabiensis*, *An. funestus* s.s., and more zoophilic, secondary vector or non-vector species in the subgenus Cellia. This information is important to understanding of the evolution of mosquito behavioral characteristics such as zoophily, anthropophily, endo- and exophagy, and endo- and exophily. Many species, such as *An. arabiensis*, are highly plastic in their behavior, and exhibit variation across the spectrum of these characteristics. As intensification of vector control measures such as insecticide-treated bed nets and indoor residual spraying with insecticides targets anthropophilic, endophagic, and endophilic species, there is the potential for shifts in the behavior of these

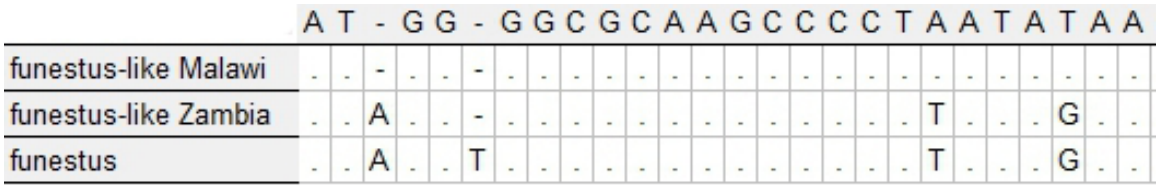

Figure 5. Variable section of Domain 3 of 28S for Malawi *An. funestus-like*, Zambian *An. funestus-like*, and *An. funestus* s.s.

species. Understanding the phylogenetic basis for these behaviors will be vital to support evidence-based vector control.

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