Morphological Assessment and Molecular Phylogenetics of the Funestus and Minimus Groups of *Anopheles* (*Cellia*)

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ABSTRACT A morphological comparison and molecular study of the Afrotropical Funestus and Afro-Oriental Minimus groups within the Myzomyia series of *Anopheles* (*Cellia*) was conducted to determine their phylogenetic affinities. Relationships were investigated using morphological characters and ribosomal (D3) and mitochondrial (COII) nucleotide sequences. Cross-identification of specimens from one group by using keys for the other group confirmed their morphological similarity, i.e., members of one group shared the key characters with members of the other group. Molecular analyses recognized five clades, not strictly related to geographical distribution: the Aconitus, Culicifacies, Funestus, Minimus, and Rivulorum subgroups. Morphological observations were congruent with the results of molecular analyses. *Anopheles leesoni*, an Afrotropical species, is closely related to the Oriental Minimus complex, and these taxa share a close relationship with the Fluviatilis complex that occurs from the Arabian Peninsula through India. The immature and adult stages of *An. rivulorum* in Africa bear morphological characters that distinguish this species from members of the Afrotropical Funestus subgroup. A composite scheme of classification based on the results and previously published information is proposed for the two groups. It is noted that *An. fluviatilis* species S is conspecific with *An. minimus* species C.

KEY WORDS *Anopheles*, 28S, COII, morphology, phylogeny

THE AFROTROPICAL FUNESTUS AND Afro-Oriental Minimus groups include morphologically similar species within the Myzomyia series of *Anopheles* subgenus *Cellia.* The Funestus group comprises eight formally recognized species: *An. aruni* Sobti, *An. brucei* Service, *An. confusus* Evans & Leeson, *An. funestus* Giles, *An. fuscivenosus* Leeson, *An. parensis* Gillies, *An. rivulorum* Leeson, and *An. vaneedeni* Gillies & Coetzee, four of which, *An. aruni*, *An. funestus*, *An. parensis* and *An. vaneedeni*, belong to the subordinate Funestus subgroup (Harbach 1994). Recently, Cohuet et al. (2003) recognized an additional "*An. rivulorum*-like" species from Cameroon. The Minimus group includes 13 species: *An. aconitus* Dönitz; *An. filipinae* Manalang; *An. flavirostris* Ludlow; *An. fluviatilis* James species S, T, and U; *An. leesoni* Evans; *An. mangyanus* Banks; *An. minimus* Theobald species A, C and E; *An. pampanai* Buettiker & Beales; and *An. varuna* Iyengar (Harbach 1994, Somboon et al. 2001). Both *An. fluviatilis* s.l. and *An. minimus* s.l. are known to be species complexes (Green et al. 1990, Subbarao et al. 1994, Somboon et al. 2001). The Culicifacies complex (Green and Miles 1980, Kar et al. 1999), *An. jeyporiensis*, and *An. majidi* are Asian members of the Myzomyia series that are not included in the Minimus group (Harbach 1994) but share uncertain affinities with the included species.

Although the Funestus and Minimus groups are regarded as separate taxa, Harrison (1980) pointed out that this was based only on geographical provenance, because the groups had not been studied jointly. Since the recognition of these groups (Gillies and de Meillon 1968, Harrison 1980), several species have been removed from one group to the other. *Anopheles fluviatilis* s.l., which occurs from the Arabian Peninsula through India, was included in the Funestus group by Gillies and Coetzee (1987), whereas Harrison (1980) had included it previously in the Minimus group. Based on cytogenetic studies, Green (1982) and Pape (1992) showed that *An. fluviatilis* s.l. and the African *An. leesoni* were more closely related to one another than to members of the Funestus group. Based on these findings, Harbach (1994) transferred *An. leesoni* and *An. fluviatilis* s.l. from the Funestus group to the Minimus group, a taxonomic act that was later supported by molecular evidence (Chen et al. 2003, Garros et al. 2005).

Few studies of mosquitoes have included combined morphological and molecular analyses (Burst et al. 1998, Manguin et al. 1999, Linton et al. 2001, Somboon

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Table 1. Species subjected to molecular and morphological analyses, source localities, and GenBank accession numbers

Taxon	Localities	28S (D3) Accession no.	COII Accession no.	Morphology	
				62-characters	6-characters
Myzomyia series					
An. culicifacies A	Pakistan, Lahore Province	AJ512728*	na	na	na
An. culicifacies B	Cambodia, Rattanakiry Province	AJ512729*	AJ512747*	na	na
An. culicifacies E	India, Ramanathapuram District	na	AJ534646*	na	na
An. jeyporiensis	China, Yunan Province	AJ512724*	AJ512743*	na	na
Funestus group					
An. aruni	Zanzibar	na	na	na	✓
An. brucei	Nigeria	na	na	na	✓
An. confusus	Zimbabwe (Rhodesia)	na	na	na	✓
An. funestus	Cameroon, Centre Province	AY259152	AY486105	✓	✓
An. fuscivenosus		na	na	na	✓
An. parensis	Kenya, Rift Valley Province	AY259155	AY486106	na	✓
An. rivulorum	South Africa, Kwazulu/Natal Pr.	AY259154	AY486107	✓	✓
An. rivulorum-like	Cameroon, North Province	AF210725*	AY727885	na	na
An. vaneedeni	South Africa, Northern Province	AY259156	na	na	✓
Minimus group					
An. aconitus	Vietnam, Khanh Hoa Province	AY259160	AY486108	na	✓
An. filipinae	Philippines, Luzon Pr.	AJ512726*	AJ512745*	na	✓
An. flavirostris	Philippines, Mindanao Pr.	AJ512723*	AJ512742*	na	✓
An. fluviatilis S	India	AF437880*	na		
An. fluviatilis T	India, Hardwar Province	AJ512734*	AJ512740*	na	✓
An. fluviatilis U	India, Hardwar Province	AJ512735*	AJ512741*		
An. leesoni	South Africa, Northern Province	AY259157	AY486109	✓	✓
An. mangyanus	na	na	U94309*	na	✓
An. minimus A	Vietnam, Hoa Binh Province	AY259158	AY486110	✓	✓
An. minimus C	Vietnam, Hoa Binh Province	AY259159	AY486111		
An. minimus E	Japan, Ryukyu Islands	AJ512751*	AJ512739*	na	na
An. pampanai	Vietnam, Khanh Hoa Province	AY259162	AY486112	✓	✓
An. varuna	Vietnam, Khanh Hoa Province	AY259161	AY486113	✓	✓

The sequences marked with an asterisk (*) were obtained from GenBank. na, no available data; Pr., province; ✓, species studied with the corresponding groups of characters.

et al. 2001, Sedaghat et al. 2003). The general objective of the present work was to elucidate relationships between and within the Funestus and Minimus groups and to assess morphological similarity on samples of species of both groups. Therefore, the relationships and group assignments of several species were questioned: the inclusion of *An. leesoni* within the Minimus group; the relationships between *An. fluviatilis* s.l., *An. leesoni*, and *An. minimus* s.l.; the position of the *An. rivulorum*-like species within the Funestus group; and the affinities of *An. culicifacies* s.l. and *An. jeyporiensis* with both groups.

The morphological work began with a novel approach whereby Asian species were run through keys for African *Anopheles*, and vice versa, to screen specimens for characters that might distinguish the Funestus and Minimus groups. Thereafter, morphological characters of the immature and adult stages were screened in species of both groups. The purpose of this study was not to investigate evolutionary relationships based on anatomical structures but merely to assess the morphological similarity of the two groups.

The distinctness of the two groups was previously partially tested by using ITS2 (internal transcribed spacer 2), D3 (domain 3), and COI (cytochrome oxidase I) markers in a cladistic analysis of 10 species (Garros et al. 2005). In the present work, molecular analyses were also done on the sequence of the D3 locus from the 28S unit of rDNA, and the amino acid sequence of the cytochrome oxidase II subunit (COII) of mtDNA. These two markers were chosen because they were the only ones available in the GenBank database that completed our set of data for a total of 22 species studied.

Materials and Methods

Morphological Data. Morphological studies were conducted on the larval, pupal, and adult stages of 17 species (Table 1), eight (of nine) of the Funestus group and nine (of 13) of the Minimus group, deposited in The National History Museum, London (list and collection numbers available upon request from CG). We examined the type specimens (holotypes, paratypes, and lectotypes) and studied specimens of each species from different parts of their distribution.

The morphological work was conducted in two steps. First, we carried out cross-identification exercises whereby the Asian species were run through morphological keys apropos for the Afrotropical Funestus group (Gillies and de Meillon 1968, Gillies and Coetzee 1987, Hervy et al. 1998), and the Afrotropical species were subjected to keys for the Oriental Minimus group (Harrison 1980, White et al. 2004). Because there is no computer key for the identification of larvae of the Asian species, the cross-identification was done using the dichotomous key of Harrison (1980). Because *An. leesoni*, an African species of the Minimus group, is included in the Afrotropical keys, we ran it through the keys for the Oriental species. We then

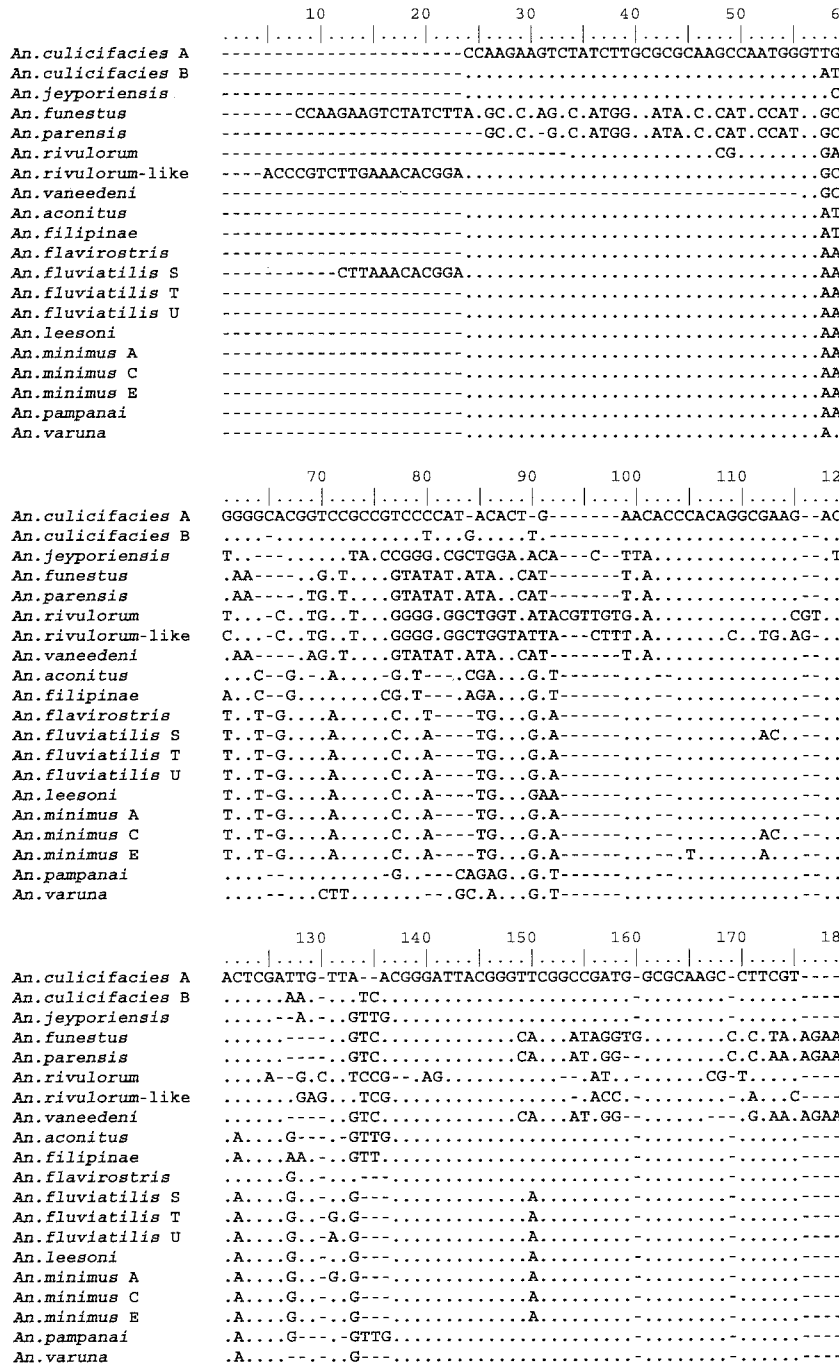

Fig. 1. 28S base alignment (347 bp).

considered some characters mentioned in the descriptions of Harrison (1980) to establish the morphological similarity of the two groups (Appendix 1). Thus, 62 potentially informative characters from the adult, larval, and pupal stages were identified and screened for six species (Table 1) used in the cross-identification exercise (*An. funestus*, *An. leesoni*, *An. minimus* s.l., *An. pampanai*, *An. rivulorum*, and *An. varuna*). Based on this initial screening, we extended the examination of the most promising characters to the 11 remaining species (Table 1). The limited number of characters observed on the all 17 species was due to either the

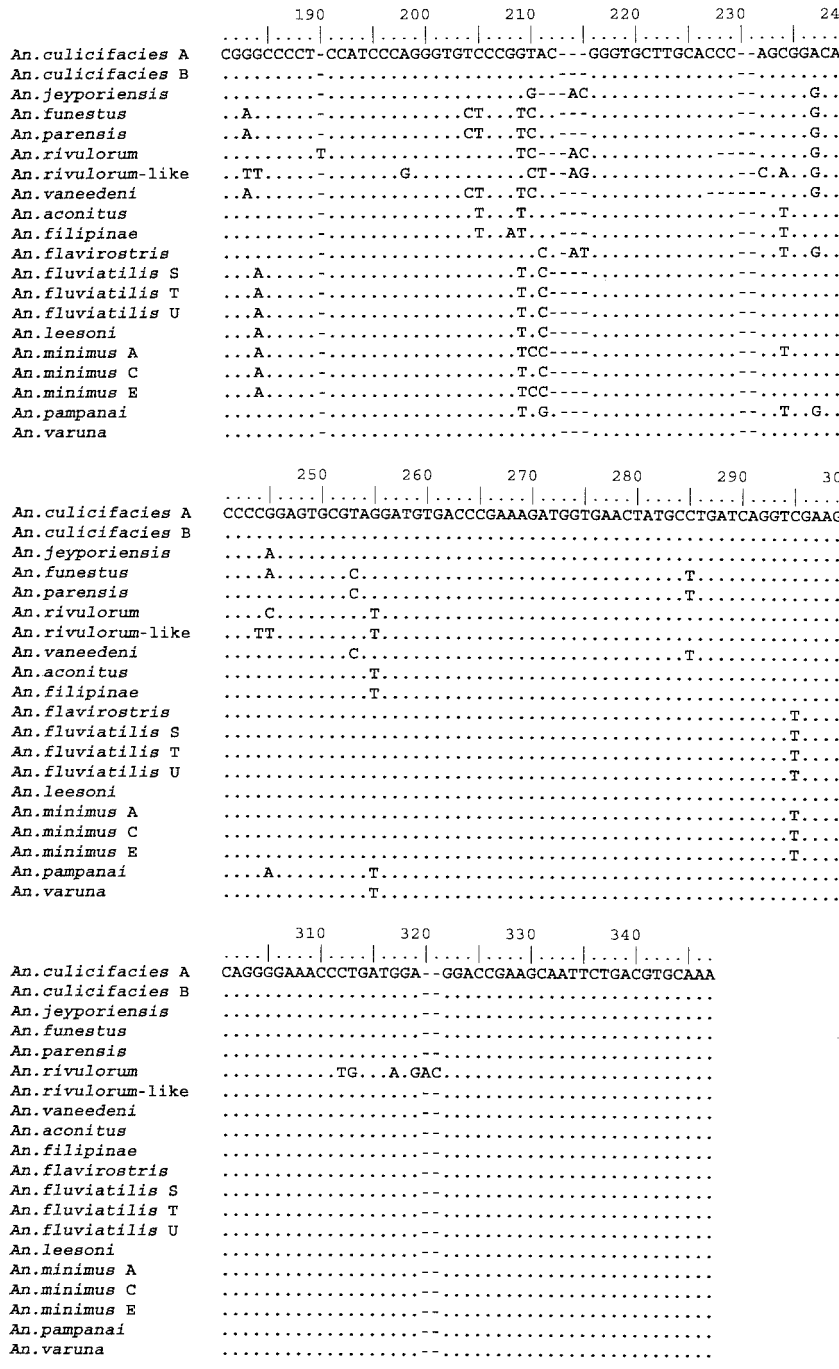

Fig. 1. (continued.)

poor condition of some specimens, leading to a difficult reading of the character state or a paucity of available specimens.

Molecular Data. The D3 sequences of the 28S unit of *An. culicifacies* species A and B; *An. jeyporiensis;* the *An. rivulorum*-like species; *An. filipinae*, *An. flavirostris*, *An. fluviatilis* species S, T, and U; *An. minimus* species E; and the COII sequences of *An. culicifacies* species B and E, *An. jeyporiensis*, *An. filipinae*, *An. flavirostris*, *An. fluviatilis* species T and U, *An. mangyanus*, *An. minimus* species E and the *An. rivulorum*-like species were obtained from the GenBank database (Table 1). Twenty other sequences (10 for D3; 10 for COII) were generated by us for *An. aconitus*, *An.*

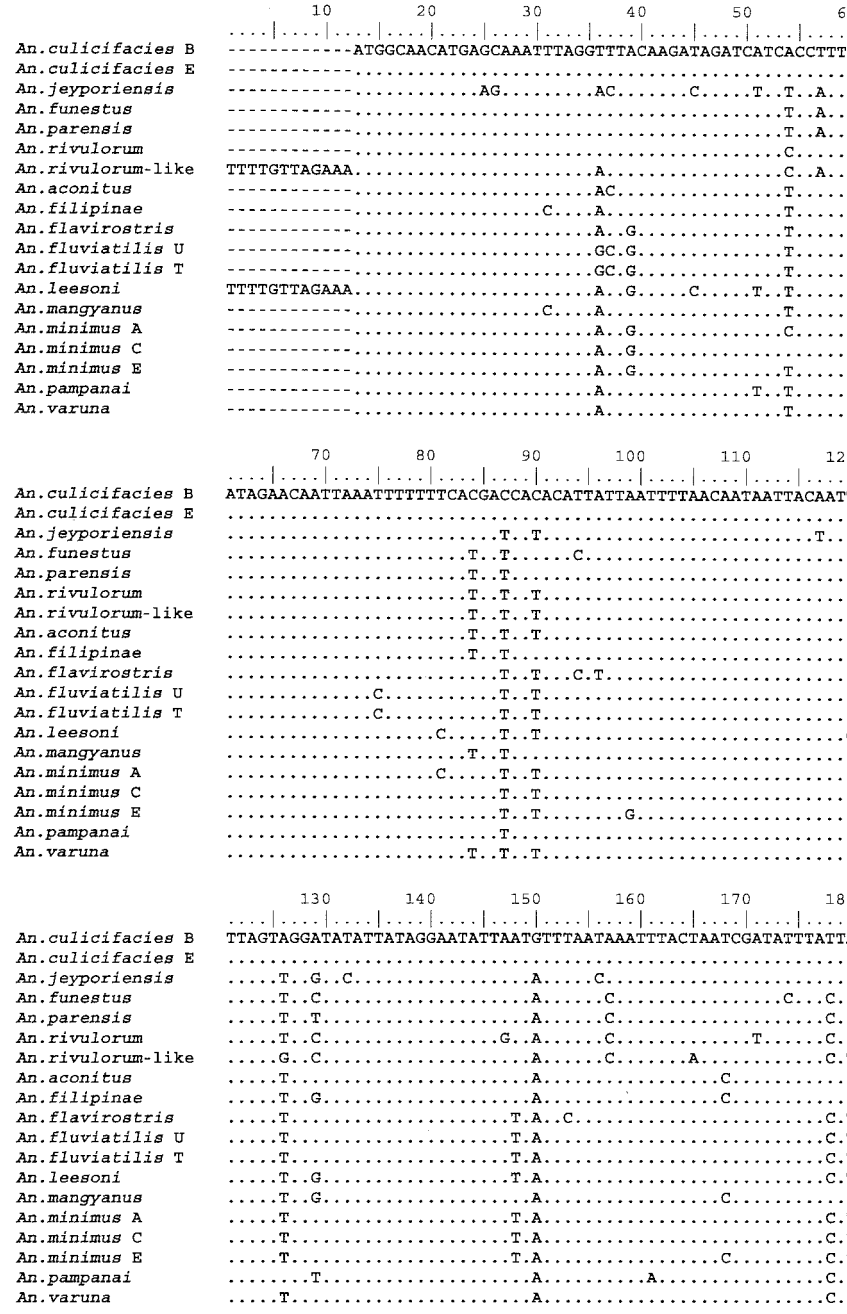

Fig. 2. COII alignment (697 bp).

funestus, *An. leesoni*, *An. minimus* species A and C, *An. pampanai*, *An. parensis*, *An. rivulorum*, *An. rivulorum*-like (COII only), *An. vaneedeni* (D3 only), and *An. varuna*.

DNA was extracted from individual dried adult mosquitoes following the protocol of Linton et al. (2001). The 28S region was amplified using the primers D3a (f) 5′ GAC CCG TCT TGA AAC ACG GA 3′ and D3b (r) 5′ TCG GAA GGA ACC AGC TAC TA 3′, and the COII subunit was amplified using the primers LEU (f) 5′ TCT AAT ATG GCA GAT TAG TGC A 3′ and LYS (r) 5′ ACT TGC TTT CAG TCA TCT AAT G 3′ (Chen et al. 2003). Polymerase chain reaction (PCR) amplification was performed in a 25-μl reaction volume containing (in final concentrations): 200 μM dNTPs, 10× buffer, 10 μM of each primer, 0.5 U of *Taq* polymerase (QIAGEN, Valencia, CA), and 2 μl of 1/10 diluted DNA extract. Thermal cycling conditions were

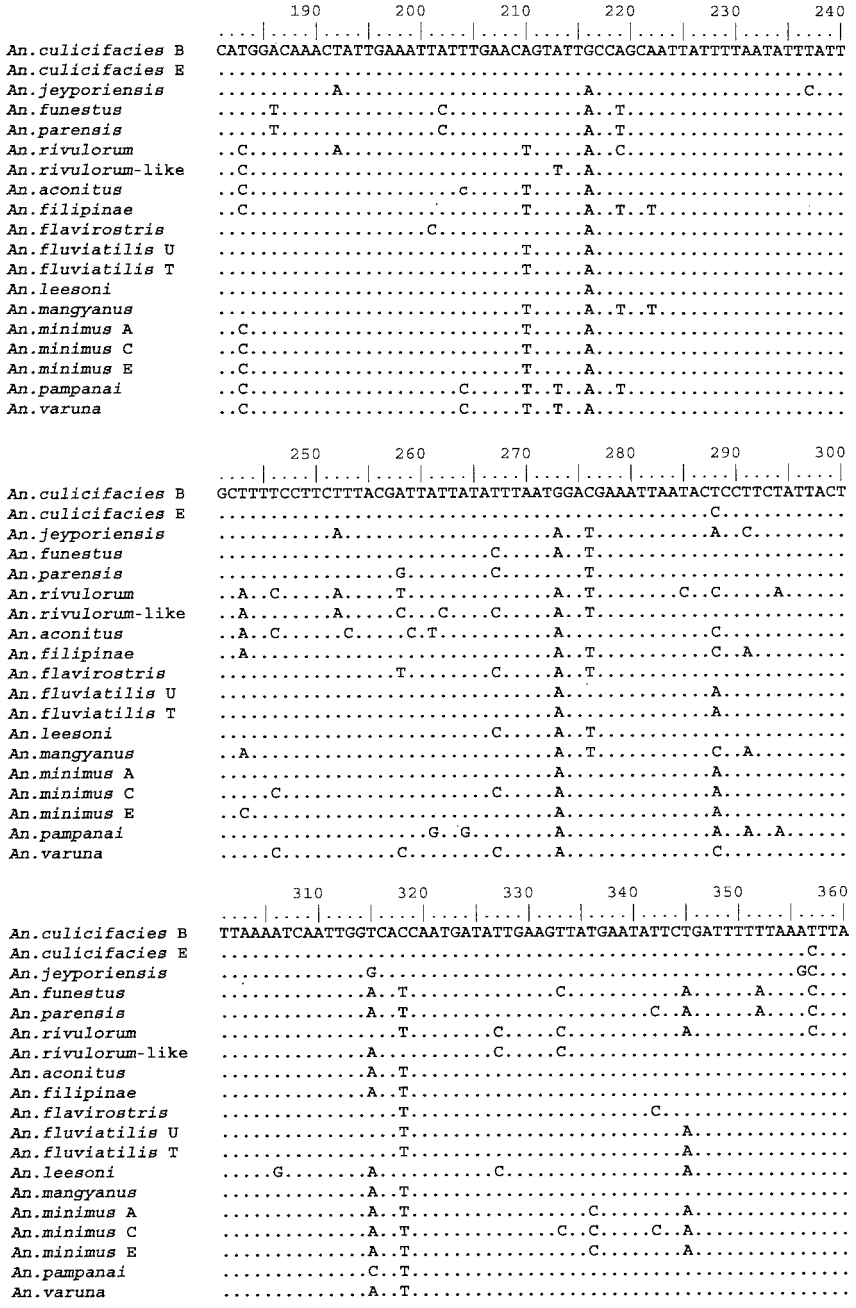

Fig. 2. (continued.)

those used by Chen et al. (2003). PCR products were checked by electrophoresis in 1.5% agarose gels containing ethidium bromide. Direct sequencing of the PCR products in both directions was done with MWG Biotech (Ebersberg, Germany). Two specimens per species were amplified and one of the two was amplified twice. Sequences are available in the GenBank database (accession numbers in Table 1).

Phylogenetic Analysis. Sequences were aligned (Figs. 1 and 2) by using ClustalW (Thompson et al. 1994). The partition homogeneity test (Farris et al. 1995) could not be used to test the incongruence between data sets because one of the two DNA sequences was missing for five species (Table 1). Basic sequence statistics were calculated with DAMBE (Xia and Xie 2001). For the protein coding COII gene, sequences were translated into amino acids by using the invertebrate mitochondrial code. Neighbor joining (NJ) and maximum parsimony (MP) analyses were conducted using PAUP*. Node support for NJ

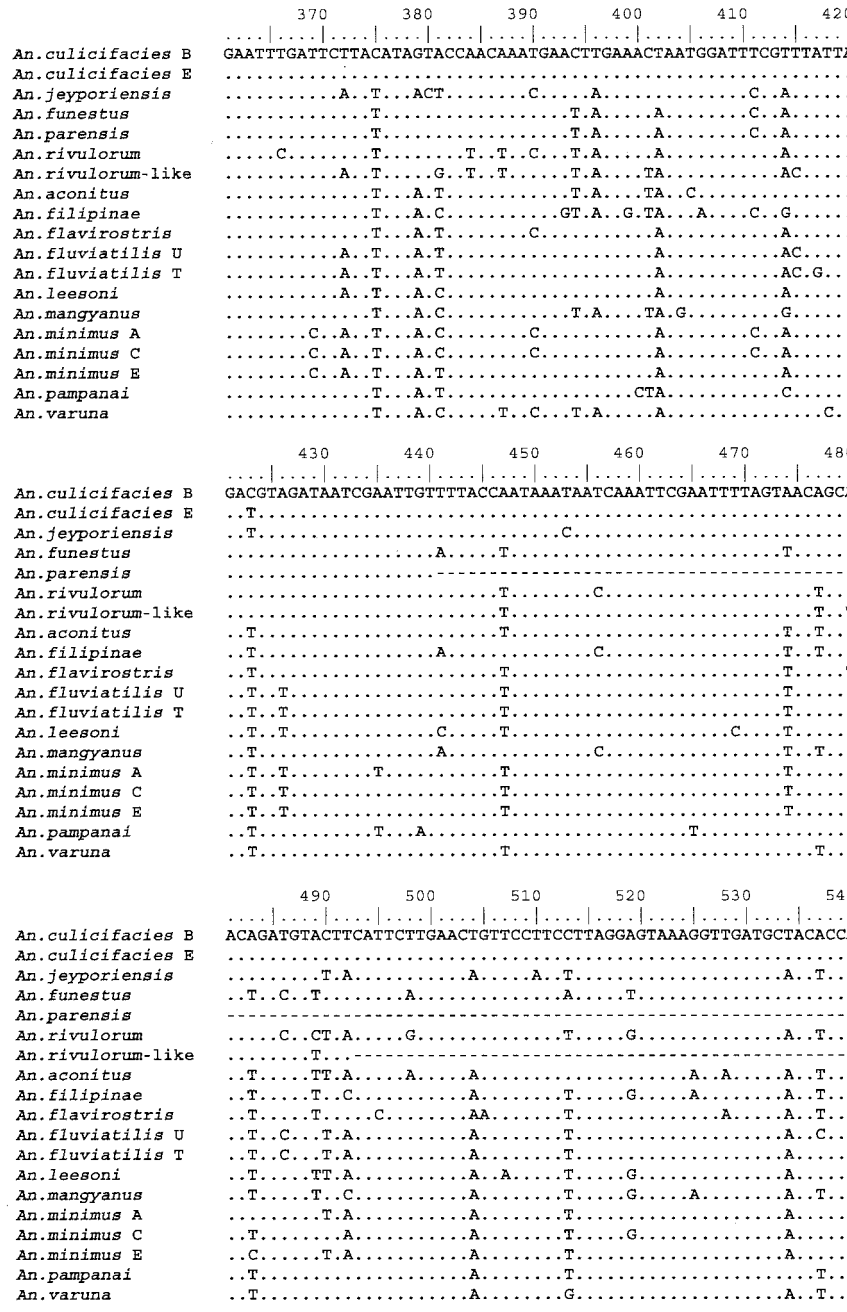

Fig. 2. (continued.)

and MP results was assessed using 1000 bootstrap pseudo-replicates. *Anopheles gambiae,* from Ivory Coast, belonging to the Pyretophorus series, was used as outgroup.

Results

Morphological Data. *Cross-Identification.* Forty-four adults and 20 larvae representing six species of the Funestus and Minimus groups were run through keys for species of the opposite group (Table 2). Species of the Funestus group were successfully identified as one or other species of the Minimus group, and vice versa. More precisely, *An. leesoni* was always identified as *An. minimus* s.l., and the latter as *An. leesoni.* Because the keys for Asian species are more detailed than the keys for Afrotropical species, the identification of the African species in keys for Asian species was more pre-

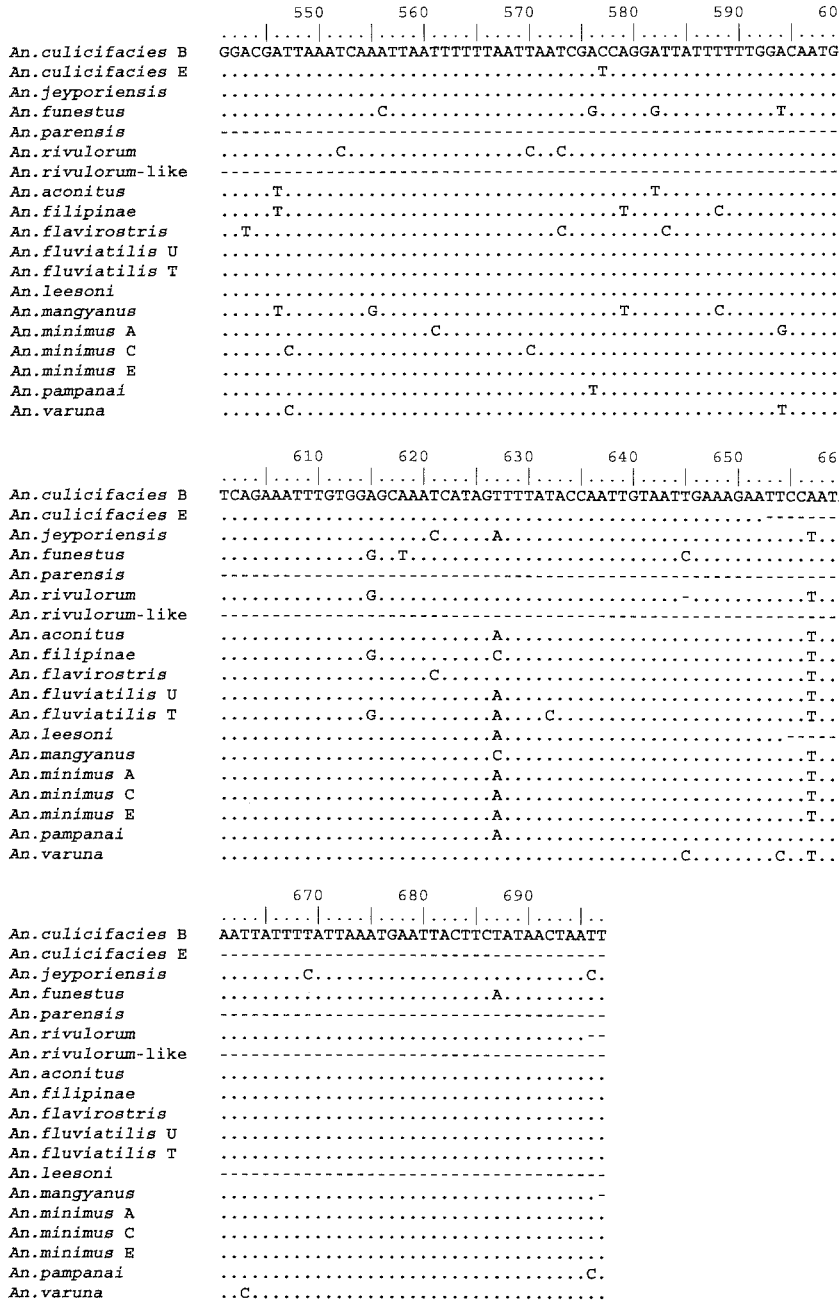

Fig. 2. (continued.)

cise. *An. rivulorum* was identified as either *An. varuna*, *An. minimus* s.l., *An. pampanai*, or *An. culicifacies* s.l., and *An. pampanai* and *An. varuna* keyed to members of the Funestus subgroup (as defined in Harbach, 1994). *An. funestus* led to *An. pampanai* or *An. varuna.*

Observations on Larvae, Pupae, and Adults. On the 62 characters observed (Appendix 1), only six were chosen for examination on the three life stages of all 17 species (Appendix 2). Small notal plates (character 17) are present on the metathorax in larvae of the five species of Minimus group and *An. leesoni* but are absent in species of the Funestus group, *An. fluviatilis* s.l., as well as *An. filipinae*, *An. flavirostris*, and *An. mangyanus*, which are island species of the Minimus group. The plates were not observed in some larvae of *An. aconitus* (12%). Thus, only species of the Minimus group present on the Southeast Asian mainland seem to possess these plates. Additionally, abdominal seta 0

Table 2. Results of cross-identification exercises whereby adults and larvae of Asian species were run through Afrotropical keys, and vice versa

Species		Identification results
An. funestus	Identified with Asian keys	Larval stage: *An. pampanai*, *An. varuna*; adult stage: *An. varuna*
An. leesoni	(Harrison 1980; White *et al.*	Larval and adult stages: *An. minimus* s.l.
An. rivulorum	2004)	Larval stage: *An. culicifacies* s.l., *An. minimus* s.l., *An. pampanai*, *An. varuna*; adult stage: *An. varuna*
An. minimus s.l.	Identified with Afrotropical keys	Larval and adult stages: *An. leesoni*
An. pampanai	(Gillies and Coetzee 1968; Hervy	Larval and adult stages: Funestus subgroup
An. varuna	*et al.* 1998)	Larval and adult stages: Funestus subgroup

(character 21) is adjacent to the edge of the anterior tergal plate in *An. flavirostris*, *An. leesoni*, and *An. minimus* s.l. The larvae of *An. leesoni* and *An. minimus* s.l. could not be distinguished. In all the other species, seta 0 is inserted on the plate (Appendix 2).

The pupal stage has been understudied despite its potential to differentiate sibling species (Harrison and Peyton 1984). *An. aruni*, *An. confusus*, *An. funestus*, and *An. parensis* have a single seta 2-Pa (character 31), whereas this seta has two or more branches in all the other species. It was difficult to be certain of the number of branches in *An. brucei* and *An. vaneedeni* because few specimens were available for study. The paddle marginal spicules (character 33) of *An. aconitus*, *An. brucei*, *An. pampanai*, and *An. rivulorum* terminate abruptly at seta 1-Pa, whereas they continue onto the inner margin of the paddle in the other species. All the African species except *An. leesoni* have setae 1,5-III (characters 37 and 38) with fewer branches than the Asian species and *An. leesoni*. These were the only two characters of the 62 screened that separated the Funestus and Minimus groups.

This illustrates the morphological similarity of the Funestus and Minimus groups. As expected from the cross-identification exercise, no key characters were unique to either group. The morphological data gave interesting results but there were too few informative characters (six) to conduct a cladistic analysis.

Molecular Phylogeny. Phylogenetic analysis of the 28S locus was carried out on the 347-character data set (accession numbers in Table 1; Fig. 1). Nucleotide frequency was not biased: T, 18.4%; C, 27.1%; A, 23.7%; and G, 30.8%. Genetic distances ranged from 43.3% between *An. mangyanus* and *An. parensis* to 1.4% between *An. fluviatilis* species S and *An. minimus* species C. When aligning only the 28S sequences of *An. flavirostris*, the Fluviatilis and Minimus complexes, and *An. leesoni*, it is noteworthy that the sequences of *An. minimus* species C and *An. fluviatilis* species S from the GenBank shared 100% of homology (Table 3).

NJ and MP analyses of the D3 data set generated the topology shown respectively in Figs. 3 and 4, which includes five clades: 1) the Minimus subgroup, with the Minimus and Fluviatilis complexes, as well as *An. flavirostris* and *An. leesoni;* 2) the Culicifacies subgroup, with members of the Culicifacies complex and *An. varuna;* 3) the Aconitus subgroup, with *An. aconitus*, *An. filipinae*, and *An. pampanai;* 4) the Rivulorum subgroup, with *An. rivulorum*, and the *An. rivulorum*-like species; and 5) the Funestus subgroup with *An. funestus*, *An. parensis*, and *An. vaneedeni*. Separation of the Aconitus subgroup from the Culicifacies subgroup is poorly supported (bootstrap values, 58%; <50%, respectively, in Figs. 3 and 4). The position of *An. jeyporiensis* remained uncertain (Figs. 3 and 4).

Sequence for the COII gene was obtained successfully for 10 of the 11 species sequenced (Fig. 2). Attempts to obtain data for *An. vaneedeni* failed (Table 1). Amplifications followed by purifications did not allow enough signal to clone or sequence the product. The gene may have mutations on the primer binding sites. *An. vaneedeni* also was difficult to sequence for a ribosomal ITS2 locus (L. L. Koekemoer, personal communication).

Of the 697 COII characters, 176 (25%) were variable between taxa, of which 112 (16%) were parsimony informative. The third codon position was much more variable (50%) than the first (13%) and second (4%) codon positions. Nucleotide frequency was biased toward A+T, averaging the greatest bias at 93.3%. Each sequence was translated into 228 amino acids where 26 substitutions were observed, of which 20 occurred in the ingroup. The NJ tree based on the COII amino acid sequences also revealed five clades (Fig. 5): 1) the Minimus subgroup, which includes the same species as the D3 tree (COII sequence was not available for *An.*

Table 3. 28S distance matrix for eight species included in the Minimus subgroup

	1	2	3	4	5	6	7	8
1. *An. flavirostris*	X							
2. *An. fluviatilis* S	0.955	X						
3. *An. fluviatilis* T	0.958	0.989	X					
4. *An. fluviatilis* U	0.958	0.989	0.996	X				
5. *An. leesoni*	0.955	0.986	0.989	0.989	X			
6. *An. minimus* A	0.958	0.982	0.993	0.989	0.982	X		
7. *An. minimus* C	0.955	**1.000**	0.989	0.989	0.986	0.982	X	
8. *An. minimus* E	0.952	0.989	0.986	0.986	0.982	0.986	0.989	X

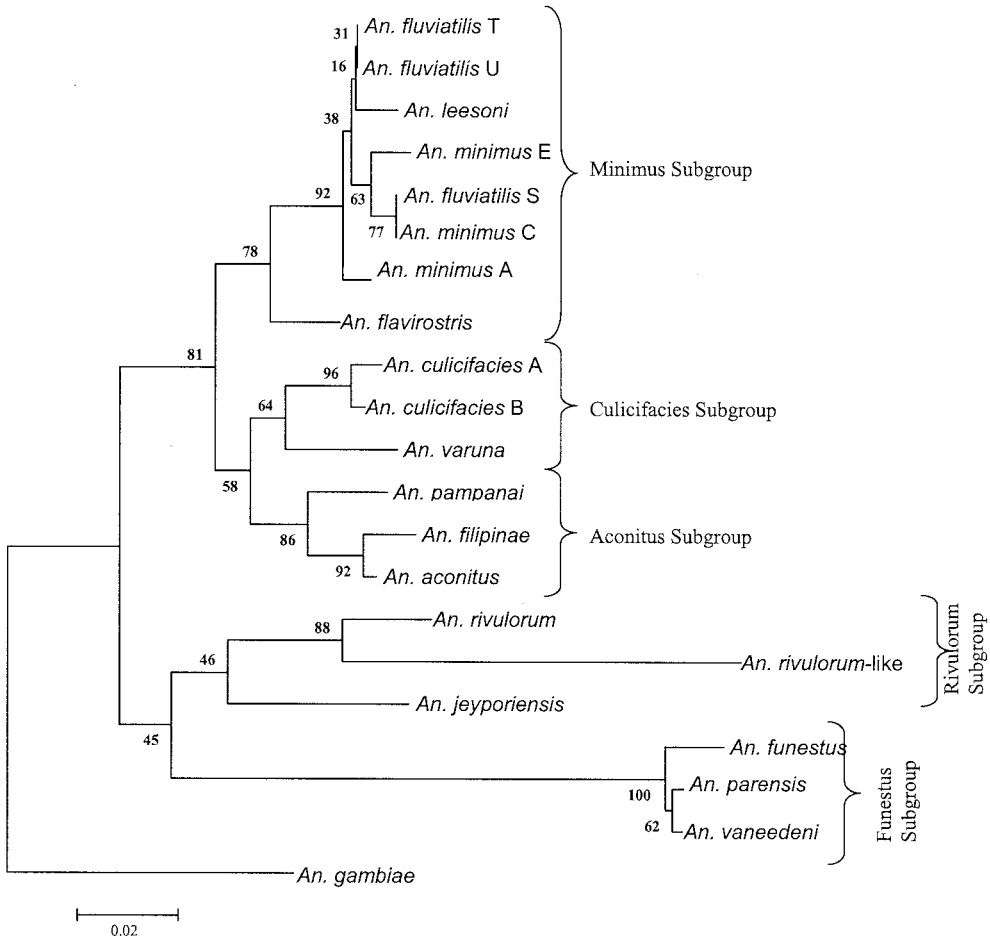

Fig. 3. Phylogenetic tree based on D3 nucleotide sequence of the 28S unit (rDNA) (NJ reconstruction).

fluviatilis species S); 2) the Culicifacies subgroup, with species B and E of the complex; 3) the Aconitus subgroup, with *An. pampanai*, *An. filipinae*, *An. mangyanus*, *An. aconitus*, and *An. varuna*; 4) the Rivulorum subgroup; and 5) the Funestus subgroup, with *An. funestus* and *An. parensis*. Differences from the D3 tree include *An. varuna* linked with the Aconitus subgroup rather than the Culicifacies subgroup, and *An. jeyporiensis* placed basal to the Minimus subgroup instead of being included with the Rivulorum subgroup. The MP tree from COII dataset (data not shown) was very similar to the NJ tree (Fig. 5), except in *An. jeyporiensis* clustering with the Rivulorum subgroup.

Discussion

The composition of both the Funestus and Minimus groups has been modified several times since and before its formal creation in 1968, and different species have been moved from one to the other group (Christophers and Puri 1931, King 1932, Christophers 1933, Gillies and de Meillon 1968, Harrison 1980). The current classification is based on morphological similarities and may not reflect actual monophyletic groups (Harbach 1994, Chen et al. 2003, Garros et al. 2005). Therefore, we conducted joint morphological and molecular studies of the two groups for the first time to examine their relationships.

Cross-identification was a novel approach for testing the similarity of species of the two groups. It revealed that differential and diagnostic characters for the Afrotropical species are shared by the Asian species, and vice versa. Consequently, recognition of separate groups based on geographical separation is not supported by the morphological data. This exercise explicitly showed that the Afrotropical and Oriental groups are indeed morphologically similar to the extent that they should not be regarded as separate taxonomic entities based merely on geographical provenance. During entomological surveys, identifications of field collected mosquitoes are mainly done on adults. There is, therefore, a need to develop a

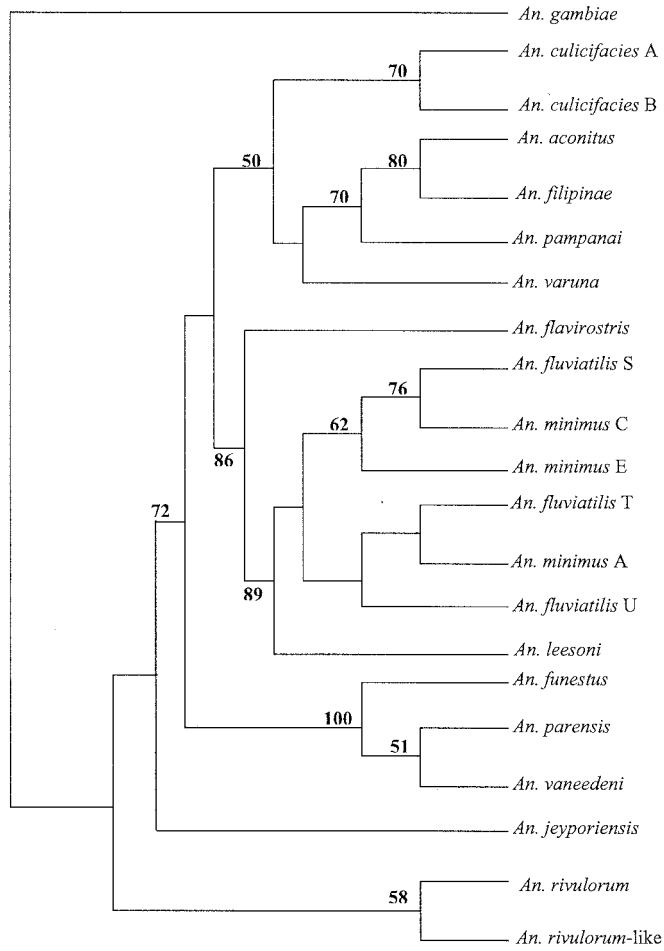

Fig. 4. Phylogenetic tree based on D3 nucleotide sequence of the 28S unit (rDNA) (MP reconstruction).

single adult identification key for members of both groups, which would help prevent the misidentification of certain sympatric species (e.g., *An. rivulorum*, *An. brucei*, and the *An. rivulorum*-like species; *An. leesoni* and *An. fluviatilis* s.l.). Combined keys also would be useful in the event that species from one region or country are introduced into another, a phenomenon that occurred with *An. gambiae* in Brazil in 1930–1940 (Soper and Wilson 1943) and in recent years with the introduction of *Aedes albopictus* Skuse into countries around the world (Grist 1993, 1994).

The molecular phylogeny, based on the D3 of rDNA and COII of mtDNA, arrayed the species of the two groups in five subgroups: the Minimus, Aconitus, Culicifacies, Funestus, and Rivulorum subgroups. Although the node separating the Aconitus and Culicifacies subgroups was poorly supported, it would be premature to unit these subgroups by including *An. culicifacies* s.l. in the Aconitus subgroup because this action is not supported by other studies (e.g., Chen et al., 2003). Chen et al. (2003) inferred the phylogenetic relationships for Oriental members of the Myzomyia series and proposed the Aconitus subgroup for the same five species as we found with the COII sequences. Thus, our results confirm the composition of the Aconitus subgroup.

The D3 and COII trees confirm the close relationships of *An. minimus* s.l., *An. leesoni*, *An. flavirostris*, and *An. fluviatilis* s.l., which comprise the Minimus subgroup. The affinities of *An. fluviatilis* s.l., *An. leesoni*, and *An. minimus* s.l. also are confirmed by the morphological data. Adults of *An. leesoni* sometimes cannot be distinguished with certainty from those of *An. funestus*, but the immature stages are distinct. The pupa of *An. leesoni* differs from all members of the Funestus group in having branched setae 2-Pa, 1-III, and 5-III, characteristics that are shared with the Minimus group. The larva of *An. leesoni* also differs from those of the Funestus group by having metathoracic plates and seta 0 adjacent to the tergal plates on segments III to VII, which are characteristics of the Minimus group. In addition, *An. leesoni* and *An. minimus* s.l. are the only two taxa that have seta 0 in the same position. In spite of its Afrotropical distribution, mor-

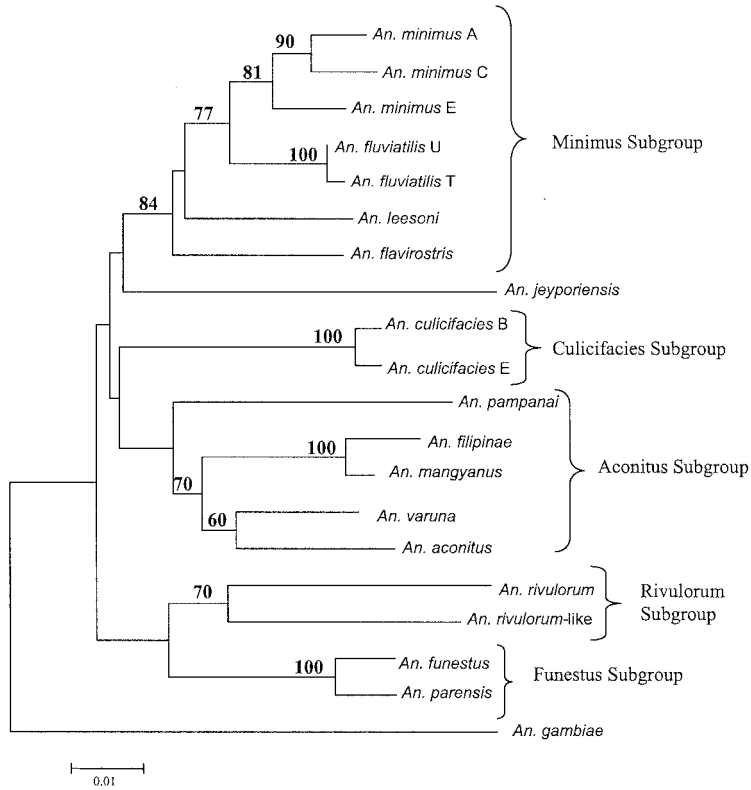

Fig. 5. Phylogenetic tree based on COII (mtDNA) amino acid sequence (NJ reconstruction).

phological (present work), cytogenetic (Green 1982, Pape 1992), and molecular studies support the position of *An. leesoni* within the Minimus group closely related to the Minimus complex (Chen et al. 2003, Garros et al. 2005). The comparison of *An. fluviatilis* s.l. with *An. leesoni* and *An. minimus* s.l. was not possible because only a few larval and pupal specimens of *An. fluviatilis* s.l. were available. However, Gillies and Coetzee (1987) reported that *An. leesoni* and *An. fluviatilis* s.l. have similar chaetotaxy, almost identical abdominal tergal plates, and possess small plates on the metathorax. *Anopheles fluviatilis* s.l. was recorded in Yemen in the southwestern corner of the Arabian Peninsula (Gillies and Coetzee 1987), but Harrison (1980) considered these records as doubtful and suggested that they might refer to another species. It is possible that these records refer to *An. leesoni.*

The 28S sequences for *An. fluviatilis* species S, T, and U from the study of Manonmani et al. (2001) were available from the GenBank database. The genetic distance between *An. minimus* C and *An. fluviatilis* S was null, indicating that they represent the same genetic species. These complexes are closely related, as indicated by <1% difference between *An. fluviatilis* T and U and *An. minimus* C. Because members of these two complexes are sympatric, species C was apparently misidentified as a member of the Fluviatilis complex, species S, rather than being recognized as the first occurrence record of the species in India. Because *An. fluviatilis* species S is obviously conspecific with *An. minimus* C, the Fluviatilis complex only include two species, T and U. This does not change the inclusion of *An. fluviatilis* s.l. within the Minimus subgroup.

An. jeyporiensis was not assigned to a subordinate group within the Myzomyia series (Harbach 1994). In the current study, this species shared a basal relationship with either *An. rivulorum* and *An. rivulorum*-like species (D3) or with the Minimus subgroup (COII). Because of these conflicting alternative associations, we decided that *An. jeyporiensis* should not be included in a subordinate group at this time. It should, however, be included in a composite group that includes all members of the Funestus and Minimus groups of Harbach (1994), which is the composite Funestus group of Garros et al. (2005). *An. majidi* was considered closed to *An. jeyporiensis* by Harrison (1980). However, because recent data on this species are missing, we found premature to include it in the composite Funestus group.

The composition of the Funestus and Rivulorum subgroups needs to be validated with the inclusion of the other species of the Funestus group. It is almost certain that *An. brucei* will fall within the Rivulorum subgroup. Harrison (1980) stated that *An. brucei* is "a small species resembling *An. rivulorum*". The pupae of *An. rivulorum* and *An. brucei* differ from the other members of the Funestus group (sensu Harbach 1994) in that the paddle marginal spicules do not extend

beyond seta 1-Pa onto the inner margin. For this reason, we suggest that *An. brucei* should be considered a member of the Rivulorum subgroup. Pape (1992) identified two clades on the basis of cytogenetic evidence, one including *An. parensis*, *An. confusus*, *An. funestus*, and *An. vaneedeni*, and the other comprised of *An. rivulorum* and *An. fuscivenosus*, which, except for the inclusion of *An. confusus*, is concordant with our results. In the absence of molecular and discernable morphological data, we think that *An. confusus* should be retained in the Funestus subgroup and *An. fuscivenosus* removed from the Funestus subgroup (Harbach 1994, Garros et al. 2005) and placed in the Rivulorum subgroup. The morphological data indicate that *An. aruni* should remain in the Funestus subgroup. Based on the results of this study, and those cited above, the African and Asian species should be included in a single group-level taxon, the Funestus group, the subordinate classification of which is shown below. Supraspecific levels of classification are denoted (as in the text above) by vernacular names formed in the manner promulgated by Belkin (1962), Peyton (1989), and Harbach (1994).

Funestus Group
- *An. jeyporiensis*
- Aconitus subgroup
  - *An. aconitus*
  - *An. filipinae*
  - *An. mangyanus*
  - *An. pampanai*
  - *An. varuna*
- Culicifacies subgroup
  - *An. culicifacies* s.l.
- Funestus subgroup
  - *An. aruni*
  - *An. confusus*
  - *An. funestus*
  - *An. parensis*
  - *An. vaneedeni*
- Minimus subgroup
  - *An. flavirostris*
  - *An. fluviatilis* s.l.
  - *An. leesoni*
  - *An. minimus* s.l.
- Rivulorum subgroup
  - *An. brucei*
  - *An. fuscivenosus*
  - *An. rivulorum*
  - *An. rivulorum*-like sp.

This study is the first to jointly investigate the Funestus and Minimus groups (sensu Harbach 1994) by using both morphological and molecular markers. It should be noted that the pupal stage showed more informative characters than the larval and adult stages. Although it is not often studied, this stage is interesting and should be studied more often. Some morphological characters of all stages seem to be useful for developing taxonomic keys to identify members of the composite Funestus group. Such studies not only improve our knowledge of anopheline taxonomy and systematics but provide a platform for investigating natural history phenomena such as vectorial capacity and making inferences about gene flow.

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Appendix 1

List of coded morphological characters examined during the study. Asterisks denote the six informative characters identified among the 17 species.

Larvae

1. Seta 4-A: fewer than five branches (0), >5 (1).
2. Seta 2-C: single (0), branched (1).
3. Seta 3-C: single (0), branched (1).
4. Seta 4-C: single (0), branched (1).
5. Seta 6-C: fewer than 10 branches (0), >10 (1).
6. Seta 8-C: fewer than five branches (0), >5 (1).
7. Seta 9-C: fewer than five branches (0), >5 (1).
8. Setae 1–2-P: arising from separate tubercles (0), arising from joined tubercles (1).
9. Seta 1-P: fewer than 20 branches (0), >20 (1).
10. Seta 2-P: fewer than 10 branches (0), >10 (1).
11. Seta 1-M: fewer than 20 branches (0), >20 (1).
12. Seta 4-M: fewer than five branches (0), >5 (1).
13. Setae 3–5-M: with small sclerotized bases (0), with large sclerotized bases (1).
14. Setae 9–10-M: short (0), long (1).
15. Seta 10-T: single (0), branched (1).
16. Seta 9-T: fewer than five branches (0), >5 (1).
17*. Notal plates of metathorax: absent (0), present (1).
18. Tergal plates: small (0), large (1).
19. Accessory tergal plates: absent (0), present (1).
20. Seta 0-III-VII: weakly developed (0), well-developed (1).
21*. Seta 0-III-VII*: on tergal plate (0), off tergal plate (1).
22. Seta 0-IV,V: fewer than five branches (0), >5 (1).
23. Seta 2-I: fewer than five branches (0), >5 (1).
24. Seta 9-I: fewer than five branches (0), >5 (1).
25. Seta 5-III: fewer than five branches (0), >5 (1).
26. Seta 13-III: fewer than five branches (0), >5 (1).

27. Seta 6-IV: fewer than five branches (0), >5 (1).
28. Seta 2-VIII: fewer than 10 branches (0), >10 (1).
29. Seta 1-X: single (0), branched (1).

Pupae

30. Seta 1-Pa: short (0), long (1).
31*. Seta 2-Pa: single (0), >2 (1).
32. Paddle marginal serrations: change gradually to spicules (0), change abruptly to spicules (1).
33*. Paddle marginal spicules: continue onto inner margin (0), end before seta 1-Pa (1), end at seta 1-Pa (2).
34. Seta 0-III-VII: short (0), long (1).
35. Seta 0-III-V: fewer than five branches (0), >5 (1).
36. Seta 0-VI,VII: fewer than five branches (0), >5 (1).
37*. Seta 1-III: fewer than 15 branches (0), >15 (1).
38*. Seta 5-III: fewer than 10 branches (0), >10 (1).
39. Seta 1-V: fewer than five branches (0), >5 (1).
40. Seta 5-V: fewer than five branches (0), >5 (1).
41. Seta 1-VI-VIII: fewer than five branches (0), >5 (1).
42. Seta 9-VII: shorter than 0.5 length of segment VIII (0), equal to 0.5 length of segment VII (1), longer than 0.5 length of segment VII (2).

Adults

43. Proboscis: scales absent at base (0), slightly erect scales present at base (1).
44. Maxillary palpus: dark erect scales absent at base (0), dark erect scales present at base (1).
45. Palpomere 5: pale (0), dark (1).
46. Dorsocentral setae: absent (0), present (1).
47. Costa: presector, sector, subcostal and preapical pale spots absent (0), these spots present (1).
48. Vein R_{2+3}, base: pale scales absent (0), pale scales present (1).
49. Vein R_2, base: pale-scaled (0), dark-scaled (1).
50. Vein R_2, apex: pale-scaled (0), dark-scaled (1).
51. Vein R_3, base: pale-scaled (0), dark-scaled (1).
52. Vein R_3, apex: pale-scaled (0), dark-scaled (1).
53. Vein M_{1+2}, at rm crossvein: pale-scaled (0), dark-scaled (1).
54. Vein M_{1+2}: pale-scaled (0), dark-scaled (1).
55. Vein CuA, base: pale-scaled (0), dark-scaled (1).
56. Vein M_{3+4}: without three dark and three pale spots (0), with three dark and three pale spots (1).
57. Vein R_2 pale fringe spot: absent (0), present (1).
58. Vein R_3 pale fringe spot: absent (0), present (1).
59. Vein 1A pale fringe spot: absent (0), present (1).
60. Vein 1A, apex: without pale scales (0), with pale scales (1).
61. Leg hindtarsomere 1: entirely dark-scaled (0), with narrow pale band (1), with broad pale band (2).
62. Leg hind tarsomere 2: entirely dark-scaled (0), with narrow pale band (1), with broad pale band (2).

Appendix 2. Summary of morphological observations for 17 species and six characters (see Appendix 1 for explanation of coding)

Species	17	21	31	33	37	38
An. aruni	0	0	0	0	0	1
An. brucei	0	0	1	2	0	0
An. confusus	0	0	0	0	0	0
An. funestus	0	0	0	0	0	1
An. fuscivenosus	0	0				
An. parensis	0	0	0	0	0	0
An. rivulorum	0	0	1	2	0	1
An. vaneedeni	0	0	1	1	0	1
An. aconitus	1	0	1	2	1	1
An. filipinae	0	0	1	1		
An. flavirostris	0	1	1	1	1	1
An. fluviatilis s.l.	0	0	1	1	1	1
An. leesoni	1	1	1	0	1	1
An. mangyanus	0	0	1	1	1	1
An. minimus s.l.	1	1	1	0	1	1
An. pampanai	1	0	1	2	1	1
An. varuna	1	0	1	0	1	1