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Molecular phylogenetics of the Oriental members of the Myzomyia Series of *Anopheles* subgenus *Cellia* (Diptera: Culicidae) inferred from nuclear and mitochondrial DNA sequences

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Abstract. The phylogenetic relationships of fifteen Oriental and two Afrotropical taxa of the Myzomyia Series of *Anopheles* subgenus *Cellia* and two outgroup species, *An. maculatus* (Neocellia Series) and *An. dirus* A (Neomyzomyia Series), were inferred from nucleotide sequences of the entire 685 bp of the mitochondrial cytochrome oxidase subunit II locus (COII) and 372 bp of the third domain (D3) of the 28S rDNA locus, both separately and together. Alignment of the D3 sequences was achieved with the aid of secondary structure comparisons, and the pattern of nucleotide substitution was best explained by the GTR + I + G model for either separate or combined datasets. Maximum likelihood and maximum parsimony analyses robustly identified five monophylies: *An. fluviatilis* U and T; *An. fluviatilis* U and T + *An. minimus* A, C, E and #157 + *An. leesoni*; *An. filipinae* + *An. mangyanus*; *An. filipinae* + *An. mangyanus* + *An. aconitus*; and *An. culicifacies* A and B. The results confirm the specific status of *An. flavirostris*, the close relationship of *An. leesoni* with the Minimus Complex, and the exclusion of *An. jeyporiensis*, *An. culicifacies s.l* and *An. funestus* from the Minimus Group. All of the species classified as members of the Minimus Group on morphological grounds formed a single clade, which comprised two subgroups: the Minimus Subgroup, including *An. minimus s.l.*, *An. fluviatilis s.l.*, *An. leesoni* and *An. flavirostris*, and the Aconitus Subgroup, including *An. filipinae*, *An. mangyanus*, *An. aconitus*, *An. pampanai* and *An. varuna*. However, these clades are only weakly supported by the present dataset.

Introduction

The Myzomyia Series of *Anopheles* subgenus *Cellia* includes sixty-four formally recognized taxa: fifty-one in the Afrotropical Region, two in the Palaearctic Region and eleven in the Oriental Region (Harrison, 1980; Harbach, 1994). The eleven Oriental taxa include *An. aconitus* Dönitz, the *An. culicifacies* Giles complex (Culicifacies Complex), *An. filipinae* Manalang, *An. flavirostris* (Ludlow), the *An. fluviatilis* James complex (Fluviatilis Complex), *An. jeyporiensis* James, *An. majidi* Young & Majid, *An. mangyanus* (Banks), the *An. minimus* Theobald complex (Minimus Complex), *An. pampanai* Büttiker & Beales and *An. varuna* Iyengar. The Minimus Complex includes three cryptic species informally designated species A, C and E (Green *et al.*, 1990; Somboon *et al.*, 2001) and a potential fourth species (form D, see Baimai, 1989; specimen #157 of Sharpe *et al.*, 2000). Subbarao *et al.* (1994) showed that *An. fluviatilis* consists of three cryptic species, designed S, T and U, based on different banding patterns of polytene chromosome arm 2. Five cryptic species are recognized within the Culicifacies Complex, referred to as species A, B, C, D and E (Subbarao, 1988; Kar *et al.*, 1999).

Most of the Oriental species of the Myzomyia Series are classified as members of the Minimus Group. *Anopheles culicifacies s.l* and *An. jeyporiensis* are unplaced within the series. Harrison (1980) pointed out that the Funestus Group

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of the Afrotropical Region and the Minimus Group of the Oriental Region comprise species that are 'so closely related that they are probably considered distinct only because of their geographical separation and the fact that no one has studied them jointly'. Consequently, *An. fluviatilis*, which reportedly extends into the Afrotropical Region in the southern part of the Arabian Peninsula (Maffi, 1971), was included in the Funestus Group by Gillies & Coetzee (1987) even though Harrison had previously classified it as a member of the Minimus Group. Based on cladistic analyses of polytene chromosome inversions, Green (1982, 1995) and Pape (1992) showed that *An. fluviatilis* and the African *An. leesoni* Evans are more closely related to one another and the Culicifacies Complex than to *bona fide* members of the Funestus Group. In light of these findings, Harbach (1994) classified both *An. fluviatilis* and *An. leesoni* as members of the Minimus Group.

Many of the Oriental species of the Myzomyia Series have been incriminated as vectors of human malarial parasites. *Anopheles minimus s.l.* is widely distributed in hilly areas throughout the Oriental Region, and is regarded as an important vector of human malaria throughout its distribution (Ho & Feng, 1958; Reid, 1968; Harrison *et al.*, 1990; Chen *et al.*, 2002). *Anopheles culicifacies s.l.* has a distribution from Ethiopia (Eritrea) eastward into southern China, Vietnam and Cambodia (Harrison, 1980; Van Bortel *et al.*, 2002) and is a vector of malaria from India and Sri Lanka westward into Iran (Harrison, 1980; Subbarao, 1988; Zaim *et al.*, 1995; Surendran *et al.*, 2000). *Anopheles fluviatilis s.l.* is a vector of malaria in India and Nepal (Subbarao *et al.*, 1994). *Anopheles aconitus* is broadly distributed from Sri Lanka, India and Nepal eastward to Hainan Island of China, and south from southern China through Southeast Asia into Indonesia (Harrison, 1980). It has been incriminated as a vector of malarial parasites in Thailand (Gould *et al.*, 1965) and is considered to be a primary vector in Java and eastern Sumatra (Damar *et al.*, 1981). *Anopheles flavirostris* occurs in the Philippines and the islands of Borneo, Java and Sumatra, and has been incriminated repeatedly, by dissection, as a vector of malarial parasites in the Philippines and East Malaysia (Harrison, 1980; Hii *et al.*, 1985). It has also been incriminated as a vector of *Wuchereria bancrofti* on Luzon and Palawan Islands and in Sabah, Malaysia (Rozeboom & Cabrera, 1964, 1965).

Knowledge about the phylogeny of the Myzomyia Series is limited. Green (1982, 1995) conducted a cladistic analysis of polytene chromosome rearrangements that included six of the Oriental taxa, *An. minimus s.l.*, *An. fluviatilis s.l.*, *An. flavirostris*, *An. aconitus*, *An. mangyanus* and *An. culicifacies s.l.* Pape (1992) reinterpreted the data of Green (1982) in his phylogenetic research involving some species of both the Myzomyia and Neocellia Series, but only included two Oriental taxa of the Myzomyia Series, *An. fluviatiluis* and *An. culicifacies s.l.* Sharpe *et al.* (2000) inferred the phylogenetic relationships between six taxa of the Minimus Group based on the cytochrome oxidase subunit II (COII) locus of mitochondrial DNA, and between four taxa of the group based on the third domain (D3) of the ribosomal 28S locus of nuclear DNA. The results of Sharpe *et al.* agreed with the phylogenetic placement of *An. fluviatilis* reported by Green (1995), but contradicted the genealogical positions he hypothesized for *An. aconitus* and *An. mangyanus*.

The use of mitochondrial DNA (mtDNA) sequence data has become standard for many phylogenetic studies (Caterino *et al.*, 2000). Among the many mitochondrial genes that have been studied, the protein-coding COII region has been extensively used for phylogenetic inference by itself or in combination with other sequences, and has proven phylogenetically informative in many insect groups (Brower, 1994; Emerson & Wallis, 1995; Smith & Bush, 1997; Maekawa & Matsumoto, 2000). The COII sequences have a consistent length of 685 bp in species of the Myzomyia Series (Sharpe *et al.*, 2000), and alignment is straightforward. Comparative analyses of nuclear DNA genes have been used to infer phylogenetic histories across a broad spectrum of taxa, from the basal lineages of life to relationships among closely related species and populations (Hillis *et al.*, 1996). The D3 domain of the 28S gene has been used as the standard for the molecular identification of species of the Minimus Group that are unreliably distinguished on the basis of morphology (Sharpe *et al.*, 1999). The D3 sequence was invariant within *An. minimus* species A and C in Thailand (Sharpe *et al.*, 2000). D3 sequences have also been used widely for phylogenetic inference in combination with other DNA sequences to obtain highly resolved phylogenies at the species level (Littlewood, 1994; Yang *et al.*, 2000; Dietrich *et al.*, 2001; O'Grady & Kidwell, 2002).

In the present study, we aimed to develop a phylogenetic hypothesis for the Oriental members of the Myzomyia Series using COII and D3 sequences to determine the evolutionary history of this group of mosquitoes and to provide a sound basis for their systematics.

Materials and methods

Taxa

The taxa investigated in this study are listed in Table 1. The ingroup comprised thirteen taxa (twelve recognized species and *An. minimus* specimen #157) of the Minimus Group, three unplaced species of the Myzomyia Series (*An. culicifacies* A and B and *An. jeyporiensis*) and *An. funestus* Giles, the nominotypical member of the Funestus Group. *Anopheles maculatus* Theobald of the Neocellia Series and *An. dirus* Peyton & Harrison (species A) of the Neomyzomyia Series, both of subgenus *Cellia,* were included as outgroup taxa.

DNA extraction, amplification and sequencing

Mosquitoes were homogenized in buffer (0.01 M Tris, pH 7.8, 0.005 M EDTA, 0.5% SDS) and digested with 50 µl/ml proteinase K at 37 °C overnight (Sambrook *et al.*, 1989). DNA was then extracted with equal volumes of

Table 1. Taxa analysed in the study, source localities and sequence accession numbers. COII sequences are all 685 bp long. The sequences marked with an asterisk (*) were obtained from GenBank. N/A indicates not successful in obtaining sequence.

Taxon	Localities	COII Accession No.	D3 Length (bp)	D3 Accession no.
Ingroup				
An. aconitus	China: Yunan, Mengla, Xiangming	AJ512744	335	AJ512725
An. culicifacies A	Pakistan: Lahore	N/A	342	AJ512728
An. culicifacies B	Cambodia: Rattanakiry, Char Ong, Char Ong Chan	AJ512747	345	AJ512729
An. flavirostris	Philippines: Mindanao, Agusan del Sur, Bayugan	AJ512742	339	AJ512723
An. filipinae	Philippines: Luzon, Bataan, Morong, Minanga	AJ512745	340	AJ512726
An. fluviatilis T	India: Hardwar, Ranipur	AJ512740	336	AJ512734
An. fluviatilis U	India: Hardwar, Ranipur	AJ512741	336	AJ512735
An. funestus	Tanzania: nr Muhesa, Zenith Village	AJ512749	362	AJ512731
An. jeyporiensis	China: Yunan, Mengla, Xiangming	AJ512743	343	AJ512724
An. leesoni	Zimbabwe: Sinoia, Hunyani River	N/A	336	AJ512736
An. mangyanus	Philippines: Luzon, Bataan	U94309*		N/A
An. minimus A	China: Yunnan, Lincang	AJ512738	336	AJ512720
An. minimus C	China: Sichuan, Qianwei, Gongping	AJ512737	336	AJ512719
An. minimus E	Japan: Ryukyu Islands, Ishigaki	AJ512739	336	AJ512721
An. minimus specimen #157	Thailand: Kanchanaburi, Ban Phu Rat	AF194461*	336	AJ512722
An. pampanai	Vietnam: Binh Thuan, Tanh Linh, Suoi Kiet	AJ512746	336	AJ512727
An. varuna	Vietnam: Binh Thuan, Tanh Linh, Suoi Kiet	AJ512748	338	AJ512730
Outgroup				
An. maculatus	China: Guangxi, Pubei, Liuyun	AJ512750	351	AJ512732
An. dirus A	China: Hainan, Baisha, Yunbang	AJ512751	367	AJ512733

phenol/chloroform/isoamyl alcohol (25 : 24 : 1) twice and chloroform/isoamyl alcohol (24 : 1) once, followed by ethanol precipitation with 0.3 M sodium acetate and 2.0 volumes of 100% ethanol on ice for 60 min. After centrifugation at 13 000 *g* for 30 min and removal of the supernatant, the pellet was washed with 70% ethanol, dried and re-suspended in 20 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) before storage at 4 °C. A negative control was included with every set of extractions.

The D3 region of the 28S gene was amplified using the primers D3a (5′-GACCCGTCTTGAAACACGGA-3′, forward) and D3b (5′-TCGGAAGGAACCAGCTACTA-3′, reverse), and the COII gene was amplified using the primers LEU (5′-TCTAATATGGCAGATTAGTGCA-3′, forward) and LYS (5′-ACTTGCTTTCAGTCATC-TAATG-3′, reverse) (Sharpe *et al.*, 2000). Amplifications were performed in 50 µl volumes overlaid with two drops of mineral oil on a HYBAID OmniGene cycler (Thermo Hybaid, Ashford, U.K.). Each PCR included 1% of the DNA of a whole mosquito, 5 µl 10 × ReddyMix™ buffer (ABgene, Epsom, U.K.), 200 µM dNTPs, 2 mM $MgCl_2$, 600 µM of each primer and 1.3 units of Thermoprime Plus DNA Polymerase (ABgene). Reactions started with denaturation at 95 °C for 5 min, followed by 35 cycles, each cycle consisting of denaturation for 40 s at 95 °C, annealing for 40 s at 55 °C and extension for 1 min at 72 °C, with a final extension at 72 °C for 6 min. PCR products were electrophoresed through ethidium bromide-stained 1% agarose gels in 1 × TBE and visualized under UV light to check for successful amplification.

PCR products were purified using Wizard PCR Prep kits (Promega Corporation, Madison, Wisconsin, U.S.A.) and sequenced in both directions using an Applied Biosystems Model 377 automated sequencer (PE Applied Biosystems, Warrington, U.K.). Sequences were deposited in GenBank with the accession numbers listed in Table 1.

Phylogenetic analysis

D3 and COII sequences were edited manually and aligned using Clustal X (Thompson *et al.*, 1997). Inference of character homology within D3 was facilitated by the identification of conserved regions based on the comparison of structural features of D3 sequences for representative eukaryotic species, including the insect species *Bombyx mori* and *Drosophila melanogaster* (Michot *et al.*, 1990), and the secondary structure predictions for the species under study using the MFold program (Walter *et al.*, 1994). For the protein coding COII gene, sequences were also translated into amino acids using the invertebrate mitochondrial code (with Transeq, European Bioinformatics Institute: http://www.ebi.ac.uk/). Basic sequence statistics were calculated with PAML (Yang, 1997) or PAUP* (version 4.0b8) (Swofford, 2001). Genetic distances between species were estimated using the best nucleotide substitution model

Table 2. Base character statistics for D3 and COII sequences across all taxa. An asterisk (*) indicates the length of the alignment that is different from the length of the individual sequences included.

Codon nucleotide position	Base composition (percent average)				Length (bp)	Percent variable sites	Percent A + T frequency
	A	C	G	T			
COII pos. 1	34.6	15.1	20.1	30.2	229	13.1	64.8
COII pos. 2	26.3	20.1	13.3	40.4	228	4.0	66.7
COII pos. 3	48.5	4.3	2.0	45.2	228	59.2	93.7
All COII sites	36.5	13.2	11.8	38.6	685	25.4	75.1
All D3 sites	24.0	28.1	29.4	18.5	372*	27.2	42.5

detected by the program Modeltest (version 3.06) (Posada & Crandall, 1998). Gaps were treated as missing data.

The data (individual and combined sequences) were tested for phylogenetic structure that is significantly different from random using the permutation tail probability (PTP) test (Faith & Cranston, 1991) with 10 000 random matrices, and randomization of ingroup taxa using PAUP*. The partition homogeneity test (Farris *et al.*, 1994, 1995), as implemented in PAUP*, was used to test for incongruence between datasets (D3 and COII) to decide whether combining the D3 and COII sequences was appropriate. The test was performed with 1000 randomly generated datasets to create a null distribution to test the statistical significance of the lengths from the original partitions.

Maximum likelihood (ML) and maximum parsimony (MP) phylogenetic analyses were conducted using the version of PAUP* noted previously. Each was performed using the heuristic search option employing step-wise addition with 100 random taxon addition sequence replicates and five trees held at each step. MP analysis was conducted either using equal weighting of transitions and transversions or a weight of 3 for transversions relative to transitions, reflecting the transition/transversion ratio estimated by maximum likelihood. The model of DNA substitution for ML was determined using the program Modeltest. Modeltest uses hierarchical likelihood ratio tests to determine which of the General Time Reversible (GTR) family of substitution models (sixty-four in all) best fits the data. Parameters (base composition, substitution rates, proportion of invariable sites and gamma shape parameter) for the chosen model were estimated by maximum likelihood (Saitou & Nei, 1987). Node support for MP analysis was assessed using 1000 bootstrap pseudo-replicates but, due to computational constraints, only 100 bootstrap replicates were performed for the ML analysis.

Results

COII sequence analysis

Sixteen taxa were sequenced successfully for the COII gene, and the COII sequence of *An. mangyanus* was obtained from GenBank (accession numbers in Table 1). Because of the age and state of preservation of the available specimens, we could not obtain COII sequence for *An. culicifacies* A or *An. leesoni*, and the sequences we determined from *An. majidi* and *An. mangyanus* appeared to be those of fungi. Each sequence for the entire COII gene of all the other taxa included in the present study has an identical length of 685 bp, and the alignment of all COII sequences was unambiguous. An adjoining 45 bp portion of the 5′tRNA-Leu gene was also amplified by the COII primers (LEU and LYS) for all species, but was excluded from further analysis. Of the 685 COII characters, 174 (25.4%) were variable between taxa, of which 107 (15.62%) were parsimony informative. As expected, the third codon position was much more variable (59.21%) than the first (13.1%) and second (3.95%) codon positions (Table 2). The PTP test indicated significant phylogenetic structure ($P = 0.0001$). Table 2 lists base character statistics for each codon position and all COII sites. Nucleotide frequency was biased toward A + T, averaging 75.08% at all codon positions, with the third codon position having the greatest bias at 93.7% A + T. The transition/transversion ratio for COII considering all codon positions is 1.79 (estimated by maximum likelihood on an NJ tree). Each COII sequence was translated to 228 amino acids with reference to the COII gene of *Drosophila melanogaster* and *An. gambiae* Giles under codon code of invertebrate mitochondria (GenBank accession numbers in Table 1). Of 228 amino acids, twenty-three substitutions were observed, of which eighteen occurred in the ingroup.

Modeltest selected the GTR model including a proportion of invariable sites and gamma distributed rate variation among sites (GTR + I + G) (Lanave *et al.*, 1984; Yang, 1994; Gu *et al.*, 1995) as the best fit for ML phylogenetic analysis of the data. The likelihood ratio (lnL = −2627.74) for the GTR + I + G model was significantly larger than that (lnL = −2635.55) of the next best model detected (GTR + G: $P = 0.000039$). The rate matrix parameters estimated were: R(a) [A-C] = 1, R(b) [A-G] = 3.13, R(c) [A-T] = 2.74, R(d) [C-G] = 0.48, R(e) [C-T] = 21.96 and R(f) [G-T] = 0. The proportions of invariable sites (I) and gamma distribution shape parameter (G) were 0.633 and 0.91, respectively. Based on the GTR + I + G nucleotide substitution model, genetic distances between species inferred from COII sequences range from 0.6% (between *An. fluviatilis* T and *An. fluviatilis* U) to 29.1% (between *An. fluviatilis* T and *An. maculatus*) (Table 3).

Table 3. Genetic distances between species based on GTR + I + G nucleotide substitution model detected by Modeltest. The distances from D3 are below the diagonal and those from COII are above the diagonal. The boldface entries indicate the minimum and maximum values, respectively. N/A indicates not available.

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1 *An. aconitus*		N/A	0.126	0.106	0.079	0.092	0.081	0.179	0.140	N/A	0.072	0.102	0.098	0.076	0.079	0.133	0.137	0.223	0.180
2 *An. culicifacies* A	0.080		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
3 *An. culicifacies* B	0.066	0.024		0.110	0.156	0.107	0.101	0.133	0.133	N/A	0.141	0.114	0.111	0.111	0.122	0.107	0.170	0.210	0.211
4 *An. flavirostris*	0.058	0.060	0.059		0.137	0.073	0.064	0.165	0.143	N/A	0.123	0.084	0.072	0.078	0.070	0.145	0.123	0.236	0.229
5 *An. filipinae*	0.023	0.078	0.055	0.078		0.136	0.132	0.169	0.168	N/A	0.013	0.140	0.125	0.130	0.133	0.152	0.179	0.271	0.245
6 *An. fluviatilis* T	0.055	0.062	0.061	0.038	0.069		**0.006**	0.163	0.117	N/A	0.131	0.039	0.041	0.030	0.040	0.122	0.162	**0.291**	0.215
7 *An. fluviatilis* U	0.055	0.057	0.057	0.038	0.074	**0.003**		0.168	0.104	N/A	0.117	0.035	0.038	0.027	0.037	0.108	0.147	0.267	0.196
8 *An. funestus*	0.184	0.203	0.200	0.174	0.175	0.194	0.202		0.227	N/A	0.169	0.184	0.166	0.174	0.170	0.218	0.207	0.255	0.270
9 *An. jeyporiensis*	0.082	0.096	0.095	0.089	0.087	0.096	0.102	0.172		N/A	0.157	0.112	0.122	0.117	0.119	0.160	0.178	0.237	0.207
10 *An. leesoni*	0.051	0.053	0.052	0.042	0.074	0.009	0.009	0.196	0.097		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
11 *An. mangyanus*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A		0.136	0.122	0.120	0.123	0.141	0.163	0.256	0.218
12 *An. minimus* A	0.051	0.067	0.066	0.035	0.065	0.006	0.009	0.201	0.101	0.016	N/A		0.025	0.025	0.032	0.121	0.161	0.275	0.207
13 *An. minimus* C	0.060	0.073	0.072	0.043	0.085	0.009	0.009	0.220	0.115	0.013	N/A	0.016		0.029	0.021	0.131	0.135	0.278	0.205
14 *An. minimus* E	0.059	0.072	0.071	0.042	0.084	0.013	0.013	0.218	0.114	0.016	N/A	0.013	0.009		0.032	0.112	0.141	0.252	0.199
15 *An. minimus* #157	0.051	0.063	0.062	0.034	0.074	0.006	0.006	0.203	0.102	0.009	N/A	0.006	0.009	0.006		0.134	0.143	0.288	0.202
16 *An. pampanai*	0.035	0.076	0.066	0.051	0.046	0.047	0.047	0.193	0.078	0.043	N/A	0.046	0.052	0.054	0.045		0.186	0.280	0.220
17 *An. varuna*	0.057	0.054	0.056	0.075	0.065	0.058	0.058	0.236	0.114	0.058	N/A	0.062	0.068	0.067	0.058	0.053		0.228	0.189
18 *An. maculatus*	0.295	0.258	0.258	0.232	0.280	0.275	0.275	0.433	0.314	0.268	N/A	0.275	0.283	0.303	0.283	0.248	0.240		0.262
19 *An. dirus* A	0.353	0.350	0.364	0.358	0.405	0.308	0.320	**0.708**	0.372	0.317	N/A	0.308	0.318	0.333	0.330	0.298	0.289	0.278	

D3 sequence analysis

Eighteen taxa were successfully sequenced for the D3 region (accession numbers in Table 1). The lengths of the D3 sequences varied from 335 bp (*An. aconitus*) to 367 bp (*An. dirus* A). The first twenty-five and last 140 bases of these sequences can be aligned unequivocally. In the central region, Michot *et al.* (1990) compared secondary structures of the D3 region across many eukaryotic species and identified four stem-loop structures that are conserved at this level despite differences in the base sequence. Three of these structures (subdomains I, III and IV) are detectable in secondary structure predictions from our sequences. The subdomains define relatively conserved stem regions (at the start and end) and less conserved loops (central parts) in the sequences. Therefore, we used the alignment of the D3 sequences of *Drosophila melanogaster* and *Bombyx mori* from Michot *et al.* (1990) as a template to guide alignment of our sequences using the Profile Alignment Mode of Clustal X. This allowed detection and alignment of the conserved, stem sections of each subdomain (see Fig. 1). The more variable residues in the loop parts of the

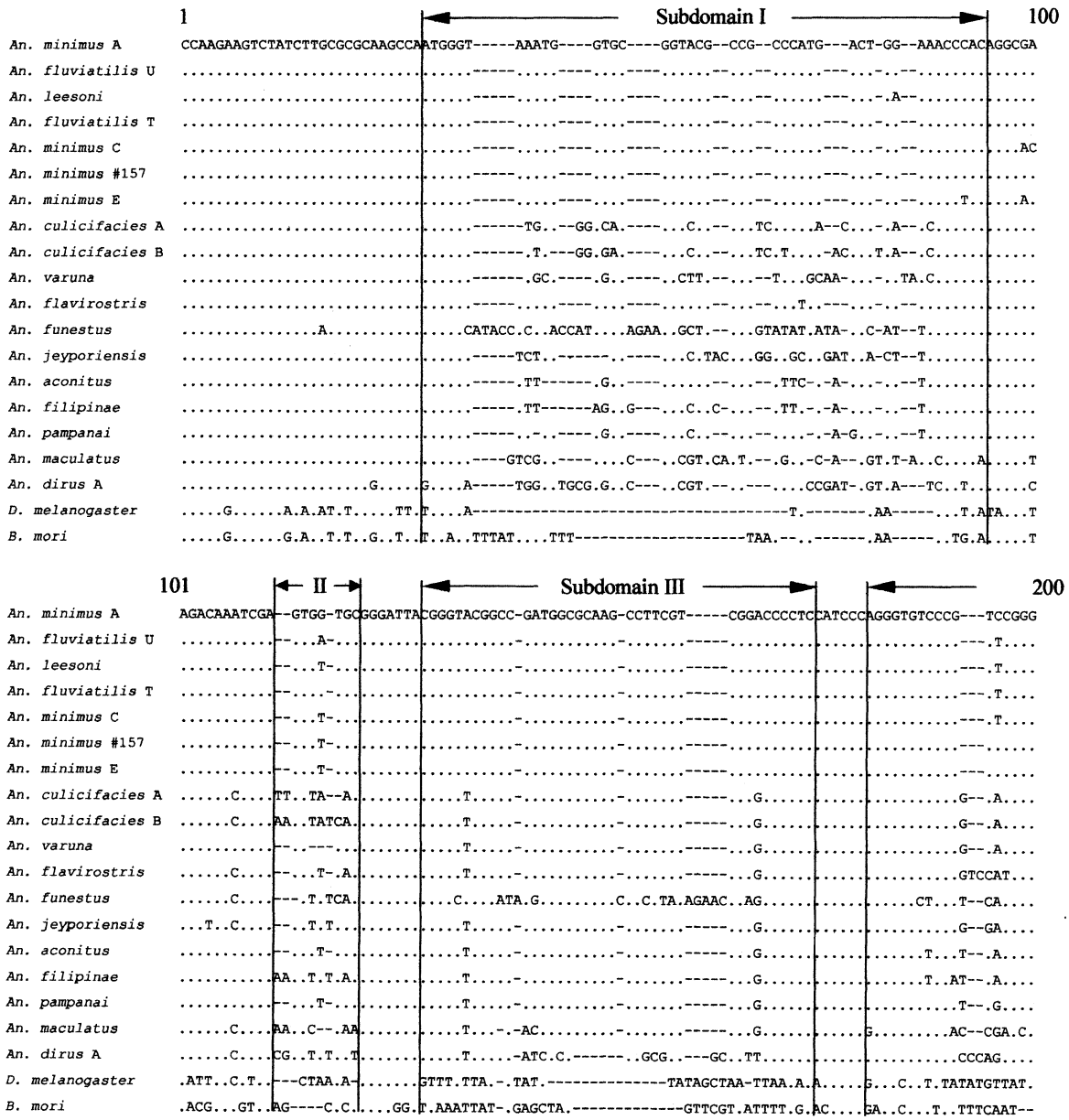

Fig. 1. Sequence alignment of the entire D3 domain of 28S rDNA. Subdomains I, II, III and IV are less conserved regions corresponding to loop structures detected by comparisons with Michot *et al.* (1990) or using Mfold (Walter *et al.*, 1994) or both (see text for details). Dots = identity; dashes = gap.

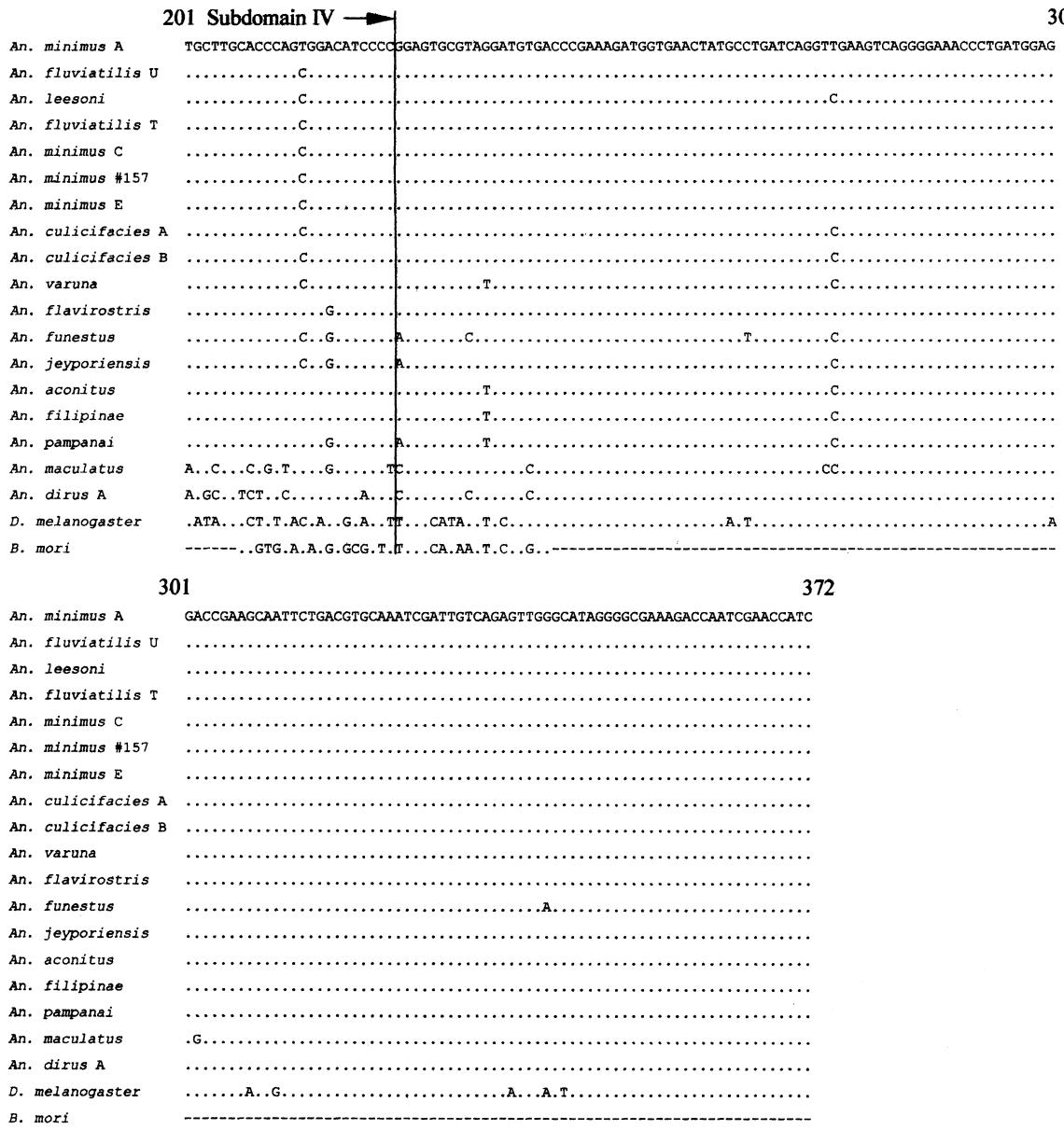

Fig. 1. Continued.

subdomains were then aligned again using the Residues Realign function of Clustal X, but these sections of the alignment remain somewhat equivocal. Subsequent phylogenetic analysis of the D3 region was carried out on the 372-character dataset (Fig. 1) after exclusion of some insertions that only occur in the outgroup sequences. Removal of these bases does not influence the reconstruction of phylogenetic relationships of species in the ingroup. The PTP test indicated significant phylogenetic structure ($P = 0.0001$). Nucleotide frequency was biased toward G + C, averaging 57.51% for all sites. The transition/transversion ratio (estimated by maximum likelihood on an NJ tree) for D3 considering all sites was 1.23.

Modeltest identified the GTR + I + G model as the best nucleotide substitution model for ML phylogenetic analysis of the D3 data. The likelihood ratio ($\ln L = -1424.87$) for the GTR + I + G model was significantly larger than that ($\ln L = -1437.68$) of the next best model (K80 + I + G: $P = 0.007$). The rate matrix parameters estimated were: R(a) [A-C] = 1, R(b) [A-G] = 1.76, R(c) [A-T] = 1.59, R(d) [C-G] = 0.15, R(e) [C-T] = 4 and R(f) [G-T] = 1.52. The proportions of invariable sites (I) and gamma distribution shape parameter (G) were 0.51 and 0.52, respectively. Based on this model, genetic distances between species inferred from D3 sequences ranged from 0.3% (between *An. fluviatilis* T and *An. fluviatilis* U) to 70.8% (between *An. funestus* T and

An. dirus A) (Table 3). We compared the D3 sequence for *An. funestus* obtained in this study with those D3 sequences (AF007094, AF007095 and AF194482) in GenBank for members of the Funestus Group, and they were similar. This confirms the identification of *An. funestus* and its surprisingly divergent D3 sequence.

Combining COII and D3 sequence

The partition homogeneity test between the COII and D3 sequences of thirteen Minimus Group taxa within the ingroup did not reject the null hypothesis of a random partition of pooled data ($P = 0.08$). Therefore, we used a combined COII and D3 dataset for subsequent phylogenetic reconstruction. The combined dataset consisted of 1057 characters, of which 275 (26.02%) were variable and 156 (14.76%) were parsimony informative. The PTP test detected significant phylogenetic structure in the combined dataset ($P = 0.0001$). Nucleotide frequencies were biased toward A + T, averaging 64.11% for all sites. The transition/transversion ratio (estimated by maximum likelihood on an NJ tree) for the combined data considering all sites was 1.40. The GTR + I + G model was also selected as the best nucleotide substitution model for ML phylogenetic analysis of the combined data. The likelihood ratio (lnL = −4216.29) for the GTR + I + G model was significantly larger than that (lnL = −4231.22) of the next best model (GTR + G: $P < 0.000001$). The rate matrix parameters estimated on the neighbour-joining tree were: R(a) [A-C] = 1, R(b) [A-G] = 3.22, R(c) [A-T] = 3.24, R(d) [C-G] = 1.39, R(e) [C-T] = 10.34 and R(f) [G-T] = 0.79. The proportions of invariable sites (I) and gamma distribution shape parameter (G) were 0.52 and 0.54, respectively.

Phylogenetic reconstruction

Maximum likelihood analysis of the combined COII and D3 dataset resulted in the topology shown in Fig. 2

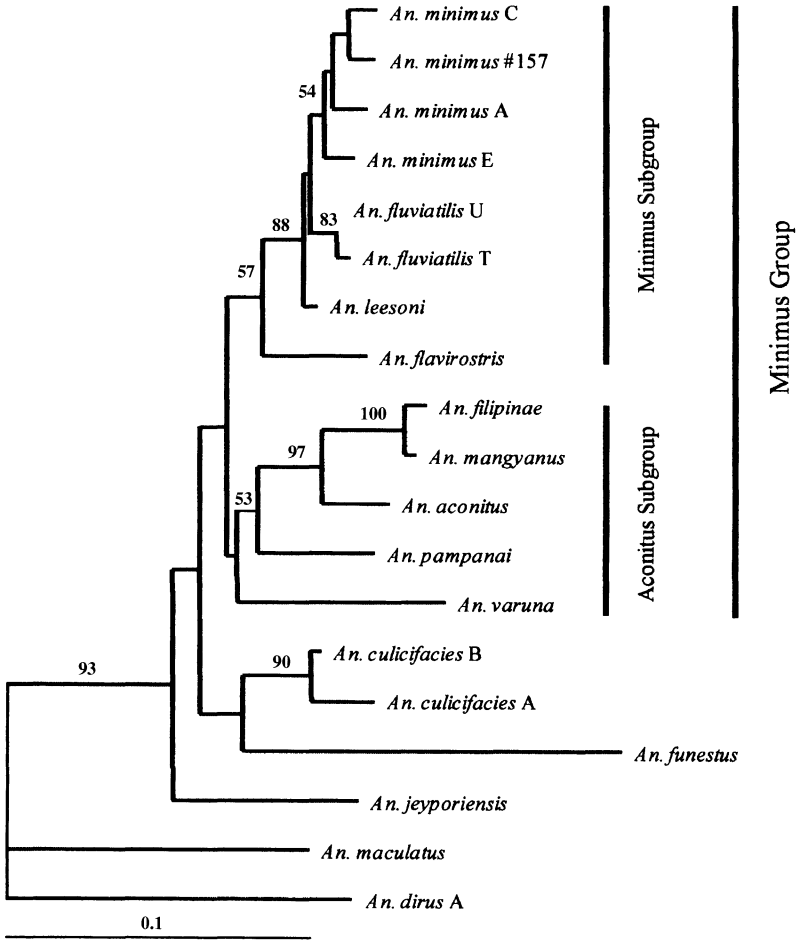

Fig. 2. Maximum likelihood tree (Ln likelihood = −4202.799) inferred from the combined COII + D3 sequences (1057 characters) under the GTR + G + I model (see Results for parameter values). The analysis shown here does not assume a molecular clock, because the simpler (clocklike) tree was rejected on a significant level of 5% based on the molecular clock likelihood ratio test. Bootstrap percentages of 100 replicates are shown above the branches where the value exceeds 50%. Branch lengths are proportional to distance.

(Ln likelihood value = −4202.79) using the GTR + I + G model described previously. Considering the transition/transversion ratio of 1.40 for the combined data, both unweighted and weighted (transversions weighted three times transitions) maximum parsimony analyses were conducted. Weighted parsimony analysis of the combined data resulted in a strict consensus tree of six equally most parsimonious trees of 1105 steps, consistency index (CI) = 0.582, homoplasy index (HI) = 0.418 and retention index (RI) = 0.537 (Fig. 3). There is no conflict between the two tree topologies: the unresolved section of the parsimony tree for the relationships among species of the Minimus Complex and *An. leesoni* are also poorly resolved by ML analysis.

When describing trees, we consider bootstrap values of 70% or greater as strong, between 50% and 70% as moderate, and below 50% as weak (Hillis & Bull, 1993). Six nodes have strong support from both analyses with at least 83% bootstrap values: Fluviatilis Complex, Fluviatilis Complex + Minimus Complex + *An. leesoni*, *An. filipinae* + *An. mangyanus*, *An. filipinae* + *An. mangyanus* + *An. aconitus*, Culicifacies Complex, and all ingroup species, respectively. Three branches received moderate support: Minimus Complex (ML analysis only), Fluviatilis Complex + Minimus Complex + *An. leesoni* + *An. flavirostris*, and *An. filipinae* + *An. mangyanus* + *An. aconitus* + *An. pampanai*.

Unweighted parsimony analysis resulted in a similar topology to weighted parsimony. The strict consensus of six equally most parsimonious trees (570 steps, CI = 0.623, HI = 0.377, RI = 0.523) differed only in that *An. varuna* was grouped with *An. jeyporiensis*. Results from the separate COII and D3 datasets using ML, weighted and unweighted MP methods, also agreed with those from the combined data in that all nodes strongly supported by the combined data were also strongly supported by the separate datasets.

Discussion

Data analysis

Despite extensive sequence divergence, reliable alignment of the D3 sequences was achieved using the comparison

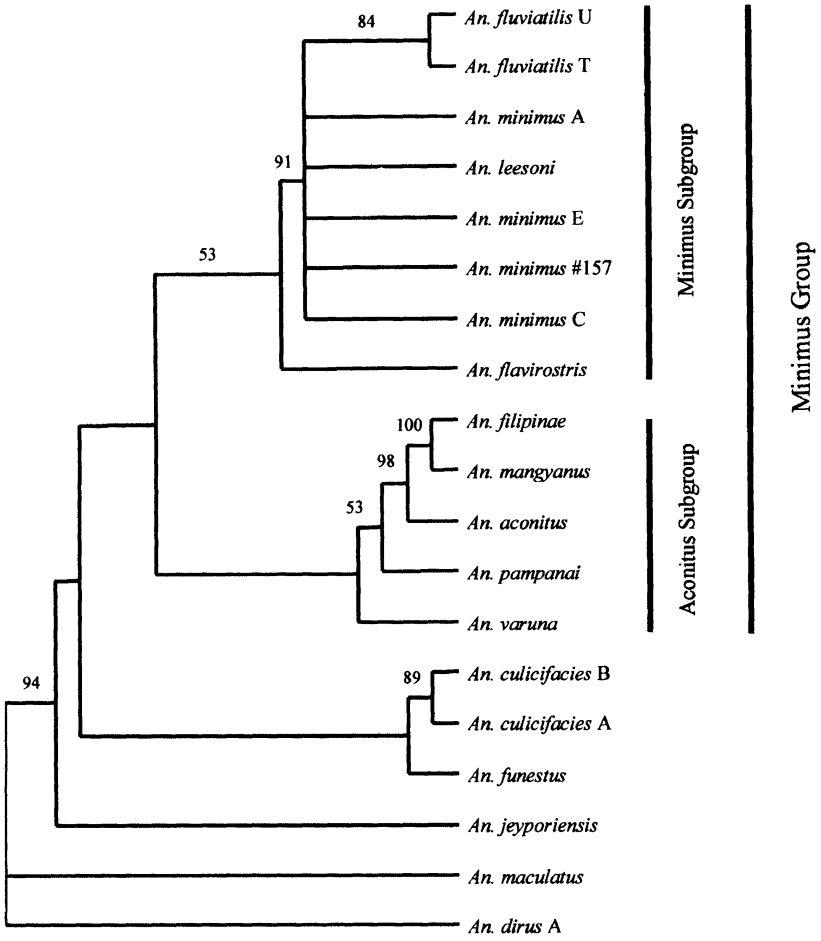

Fig. 3. Strict consensus of six equally most parsimonious trees (1105 steps, see Results for parameter values) inferred from the combined D3 and COII sequences (1057 characters). Transversions weighted three times as heavily as transitions. Bootstrap percentages of 1000 replicates are shown above the branches where they exceed 50%. Branch lengths are not arbitrary.

with other eukaryotic D3 sequences and secondary structure predictions to identify conserved residues (Fig. 1). Alignment of loop sections of the four subdomains remained difficult. Therefore, we repeated the phylogenetic analyses with these sections excluded. This did not cause any change in the topography of the trees. Separate and combined COII and D3 datasets all showed significant phylogenetic structure with the PTP test, confirming the utility of these two loci for phylogenetic reconstruction of the taxa examined. The two loci showed similar patterns of substitution (following the GTR + I + G model) and did not differ more than random partitions of the data. Therefore, it was possible to combine them in both ML and MP analyses. The likelihood and weighted parsimony analysis for the combined data produced fully compatible topologies (Figs 2, 3). The combined dataset maximizes the amount of information available and is most likely to yield the correct tree (Vogler & Welsh, 1997; Chippindale *et al.*, 1999). Many authors have stressed the need to substantiate phylogenetic relationships inferred from single loci with independent loci (Pamilo & Nei, 1988; Avise, 1989) because a phylogeny based on a single locus may deviate substantially from the true species tree. Recently, an increasing number of phylogenetic studies have combined nuclear and mitochondrial DNA sequences (Clark *et al.*, 2001; Damgaard & Sperling, 2001; Johnson *et al.*, 2001; Edgecombe *et al.*, 2002; O'Grady & Kidwell, 2002). Congruence between such loci increases confidence in the resulting tree, as in this case. However, resolution of some relationships among the Oriental members of the Myzomyia Series requires more data, ideally from additional loci, as well as the inclusion of species (*An. majidi* and *An. culicifacies* species C and D) that could not be obtained for this study.

Phylogenetic relationships and systematics

All analyses based on either COII, D3 or the combined dataset suggested the existence of the following monophylies: (1) Fluviatilis Complex, (2) Fluviatilis Complex + Minimus Complex + *An. leesoni*, (3) *An. filipinae* + *An. mangyanus*, (4) *An. filipinae* + *An. mangyanus* + *An. aconitus*, (5) Culicifacies Complex, and (6) all ingroup taxa (Figs 2, 3). The combined data from all three analyses also moderately supported clades including *An. flavirostris* and the Minimus Complex, and *An. pampanai* and *An. aconitus*, *An. mangyanus* and *An. filipinae*. ML analysis of the combined data and MP analyses (both weighted and unweighted) of the COII data supported the monophyly of the Minimus Complex, but only weakly.

The Afrotropical *An. leesoni* was thought to be a member of the Funestus Group by Harrison (1980). Green (1982, 1995) suggested its close relationship with *An. minimus s.l* and Harbach (1994) moved the species to the Minimus Group. The present study confirms its close relationship with the Minimus and Fluviatilis Complexes (these three forming a strongly supported monophyly). The species of the Funestus Group are very important vectors of human malarial, filarial and arboviral pathogens in Africa. The complex was thought to be so closely related to the Oriental Minimus Group that these two species assemblages were probably considered distinct only because of their geographical separation (Harrison, 1980). Green (1982, 1995) and Pape (1992) suggested a more distant phylogenetic relationship with the Minimus Group, which has been confirmed in this study. *Anopheles flavirostris* was considered to be a subspecies of *An. minimus s.l.* (King, 1932; Reid, 1968) until Harrison (1980) recognized it as a separate species on the basis of morphological differences. Somboon *et al.* (2000) supported the specific status of *An. flavirostris* by hybridization experiments with *An. minimus* A and E, and fine structures of female cibarial armature observed by scanning electron microscopy. Our study further supports its specific status and demonstrates its close relationship with the Minimus Complex. Harbach (1994) did not assign the Culicifacies Complex or *An. jeyporiensis* to any of five named groups in the Myzomyia Series. Our data reveal their quite large genetic distance from the Minimus Group, and the Culicifacies Complex appears to be closer to *An. funestus*, with *An. jeyporiensis* possibly forming an independent, basal branch.

Green (1982, 1995) constructed a phylogeny using cladistic analysis for some members of the Myzomyia Series based on inversions observed in ovarian polytene chromosomes. His studies included *An. minimus s.l.*, *An. fluviatilis s.l.*, *An. flavirostris*, *An. aconitus*, *An. mangyanus* and *An. culicifacies s.l.* Our results concur with his, except on two points: (1) *An. flavirostris* does not have a closer relationship with *An. minimus s.l* and *An. fluviatilis s.l.* than it does with *An. leesoni*, and (2) the Culicifacies Complex does not have a closer relationship with the Minimus Group than with *An. funestus*. Pape (1992) constructed a phylogeny for some species of the Myzomyia and Neocellia Series, including the data of Green (1982). The phylogenetic relationships suggested for *An. fluviatilis s.l.*, *An. leesoni*, *An. culicifacies s.l* and *An. funestus* were the same as those of Green (1982). The ML tree of D3 sequences constructed by Sharpe *et al.* (2000) for *An. minimus* A, C and #157, *An. aconitus*, *An. varuna*, *An. flavirostris*, *An. jeyporiensis* and *An. funestus* is congruent with the D3 topologies on the basis of ML and MP algorithms produced here with additional data. However, the COII ML tree of Sharpe *et al.* (2000) for *An. minimus* A, C and #157, *An. aconitus*, *An. varuna*, *An. flavirostris*, *An. filipinae*, *An. mangyanus* of the Minimus Group, and *An. hilli* Woodhill & Lee of the Neomyzomyia Series, is different from our COII likelihood tree to some extent. We used equal weighting of transitions and transversions for ML analysis and GTR + I + G model, whereas Sharpe *et al.* (2000) used a weight of 2 for transversions relative to transitions, and possibly a simple model (not stated). This may account for the observed differences.

Harrison (1980) assigned all Oriental species of the Myzomyia Series, except *An. culicifacies s.l.*, *An. jeyporiensis* and *An. majidi*, to the Minimus Group. He stated that the eight species in this group (at his time) shared derived

morphological features in nearly all life stages, which was evidence that the Minimus Group is a fairly old assemblage. Subsequently, the cryptic species of the Minimus Complex (A, C, E and #157) (Green *et al.*, 1990; Sharpe *et al.*, 2000; Somboon *et al.*, 2001) and the Fluviatilis Complex (S, T and U) ((Subbarao *et al.*, 1994) were reported, and, more importantly, Harbach (1994) moved the Afrotropical *An. leesoni* into the group. Our results support this morphological classification. Green's (1995) analysis of ovarian polytene chromosome inversions separated six members of the Minimus Group into two clades: *An. minimus s.l.* + *An. fluviatilis s.l.* + *An. leesoni* + *An. flavirostris*, and *An. aconitus* + *An. mangyanus*. Our data also support this division. Although these clades are not strongly supported by the DNA sequence data alone, the correspondence with morphological and cytogenetic differences suggests that they are veritable descendent groups. *Anopheles varuna* is the only species whose placement remains in doubt.

Detailed taxonomy can provide a means of communicating about biological entities and associating taxa with ecological and behavioural data. Therefore, there is a need to give separate names to each of these two well defined clades. Among these species, *An. minimus s.l.* is regarded as an important vector of human malaria throughout its distribution in the Oriental Region (Ho & Feng, 1958; Reid, 1968; Harrison *et al.*, 1990), and *An. aconitus* in Java, eastern Sumatra and Thailand (Damar *et al.*, 1981). For this reason, we define here the former clade as the Minimus Subgroup and the latter as the Aconitus Subgroup (Figs 1, 3).

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