SYSTEMATICS

Phylogeny of the Leucosphyrus Group of *Anopheles* (*Cellia*) (Diptera: Culicidae) Based on Mitochondrial Gene Sequences

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ABSTRACT We evaluated fragments of the mitochondrial COI and ND6 genes to explore phylogenetic relationships among 13 of the 20 species of the Leucosphyrus Group of *Anopheles* (*Cellia*) (Diptera: Culicidae), including all four of the currently recognized complexes. Nucleotide sequence data were analyzed using maximum parsimony, maximum likelihood, and Bayesian methods. The results revealed the monophyly of the Leucosphyrus Group and the Hackeri and Riparis Subgroups; however, the Leucosphyrus Subgroup and the Leucosphyrus Complex were recovered as polyphyletic. The monophyly of the Dirus Complex was corroborated by all the analyses but with discordance in the placement of *An. balabacensis* Baisas. The maximum parsimony strict consensus tree and maximum likelihood topology support the placement of *An. balabacensis* within the Dirus Complex, whereas the Bayesian topology placed the species as sister to the Hackeri and Riparis clade. Support for the split leading to *An. latens* Sallum & Peyton and *An. leucosphyrus* Dönitz is not strong; however in the maximum likelihood topology by using PHYML, they were recovered in a basal group within the Leucosphyrus Group.

KEY WORDS *Anopheles*, Leucosphyrus Group, phylogeny, COI, ND6

The Leucosphyrus Group belongs to the Neomyzomyia Series of *Anopheles* (*Cellia*) (Diptera: Culicidae) (Harbach 2004; Sallum et al. 2005a,b) and includes 20 named species and two geographical forms (Peyton 1989). Six species of the Leucosphyrus Group are of great epidemiological importance as highly competent vectors of human malaria parasites in Southeast Asia: *Anopheles balabacensis* Baisas (White 1983, Schultz 1992, Barcus et al. 2002), *Anopheles latens* Sallum & Peyton (Zulueta 1956, White 1983), *Anopheles leucosphyrus* Dönitz (Warren et al. 1963), *Anopheles baimaii* Sallum & Peyton (Rahman et al. 1977, Rosenberg and Maheswary 1982, Dutta et al. 1991, Prakash et al. 2001), *Anopheles dirus* Peyton & Harrison (Eyles et al. 1964; Scanlon and Sandhinand 1965; Sloof and Verdrager 1972; Ismail et al. 1974, 1975; Wilkinson et al. 1978; Deng et al. 1982; Trung et al. 2004), and *Anopheles sulawesi* Koesoemawinangoen (Warren and Wharton 1963). Other species of the group are suspected to transmit simian malaria parasites (Warren and Wharton 1963, Coatney et al. 1971, Tsukamoto et al. 1978, Fooden 1994).

The current classification of the Leucosphyrus Group was initially proposed by Colless (1956) and Reid (1968) and later corroborated by Peyton (1989). Subsequently, Peyton proposed the Elegans, Leucosphyrus and Riparis Subgroups based on morphological similarities. The Leucosphyrus Group was demonstrated to be monophyletic and the earliest diverged lineage within the subgenus *Cellia* (Sallum et al. 2000). Species of the Leucosphyrus Group were defined mainly based on morphology (for details, see Sallum et al. 2005b), but the 12 species included in the Leucosphyrus Subgroup, plus *An. mirans* Sallum & Peyton (Hackeri Subgroup), were investigated using a multidisciplinary approach that included morphology (Peyton and Harrison 1979, Peyton and Ramalingam 1988), karyotypes, polytene chromosomes, and crossing studies (Baimai et al. 1984a,b, 1987, 1988a,b,c; Baimai and Green 1985; Sawadipanich et al. 1990; Poopittayasataporn and Baimai 1995). Consequently, to distinguish among the species it is necessary to use all life stages (Sallum et al. 2005a,b), ultrastructure of the eggs (Damrongphol and Baimai 1989), and alternative identification methods such as those of Baimai et al. (1987, 1988b,c), Sawadipanich et al. (1990), Walton et al. (1999), Huong et al. (2001), and Manguin et

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al. (2002). The Leucosphyrus Subgroup includes the Dirus Complex, the Leucosphyrus Complex, the unassigned *Anopheles baisasi* Colless, and the geographical Con Son Form. The Dirus Complex comprises seven species, *An. dirus*, *Anopheles cracens* Sallum & Peyton, *Anopheles scanloni* Sallum & Peyton, *An. baimaii*, *Anopheles nemophilous* Peyton & Ramalingam, *Anopheles elegans* (James), and *Anopheles takasagoensis* Morishita. The Leucosphyrus Complex includes *An. leucosphyrus*, *An. latens*, *Anopheles introlatus* Colless, and *An. balabacensis*. The Riparis Subgroup consists of *Anopheles riparis* King & Baisas, *Anopheles cristatus* King & Baisas, *Anopheles macarthuri* Colless, and the Negros Form. Sallum et al. (2005a) transferred *An. elegans* to the Dirus Complex and thus renamed the Elegans Subgroup as the Hackeri Subgroup to reflect the change. Currently, the Hackeri Subgroup includes *An. hackeri* Edwards, *An. pujutensis* Colless, *An. mirans*, *An. sulawesi*, and *Anopheles recens* Sallum & Peyton.

Several studies using genetic and molecular tools were carried out to investigate species recognition, gene flow, and genetic population structure of members of the Leucosphyrus Group (for review, see Sallum et al. 2005b), but few studies have addressed phylogenetic relationships among members of the Dirus Complex. Crossing experiments (Baimai et al. 1987), cytological studies (Baimai et al. 1980, 1988c), and allozyme analysis (Green et al. 1992) all suggested a sister relationship between *An. dirus* and *An. scanloni*, with *An. baimaii* being more distantly related. In contrast, Walton et al. (2000, 2001) found that *An. dirus* and *An. baimaii* are genetically more similar to each other than to *An. dirus* or *An. scanloni*. More recently, Manguin et al. (2002) observed that *An. scanloni* shares sequence characterized amplified regions (SCAR) fragments with *An. dirus*.

The objectives of this study were 1) to test the monophyly of the Leucosphyrus Group; 2) to test the monophyly of the Leucosphyrus, Riparis, and Hackeri Subgroups; and 3) to estimate phylogenetic relationships among taxa of the Leucosphyrus Group. Two geographical Forms, Con Son and Negros, were not included, nor were seven other species for which we could get neither fresh specimens nor DNA from museum specimens.

Materials and Methods

Collection data are in Table 1. In this study, we used fragments of the mitochondrial cytochrome *c* subunit I (COI mtDNA) and the NADH dehydrogenase subunit six genes (ND6 mtDNA) derived from museum specimens. Leucosphyrus Group species identifications were confirmed by either morphology or polytene chromosomes (for details, see Sallum et al. 2005b). All specimens are deposited in the Smithsonian Institution, National Museum of Natural History (NMNH) collection. Most adult specimens were individually reared with fourth instar larval and pupal exuviae, and adult male genitalia kept as vouchers. When possible, we used progeny brood specimens that originated from individual wild-caught adult females subsequently identified by V. Baimai by using polytene chromosomes (for details, see Sallum et al. 2005b). The remaining individuals are stored dry in the NMNH where they remain at ambient temperature. DNA vouchers are stored at −80°C at NMNH.

DNA Extraction, Polymerase Chain Reaction (PCR) Purification, and Sequencing. Total DNA was extracted using the DNeasy tissue kit (QIAGEN, Valencia, CA) following the manufacturer's animal tissue extraction protocol. DNA template was eluted in 50 μl of buffer AE. Because the chance of cross-contamination is high when using museum specimens, we used negative controls for both DNA extractions and PCRs. The two primers used to amplify 250 bp of the COI gene were UEA9.2 (5′-CTA ACA TTT TTT CCT CAA CAT TTT TTA GG-3′) and UEA10.2 (5′-TTA TTA GTT AAT AAY GGT ART TCT G-3′), both designed for this study. PCR reactions were carried out in a total volume of 50 μl by using standard protocols (Palumbi 1996). PCR amplification profile consisted of 2 min at 95°C, five cycles of 1 min at 94°C, 40 s at 37°C and 40 s at 72°C, followed by 45 cycles of 40 s at 94°C, 40 s at 48°C, and 40 s at 72°C. PCR amplification was terminated with an extension of 7 min at 72°C. The two primers used to amplify 349 bp of the ND6 gene were ND6.F2 (5′-TTG GWC GTA AWG GWC CAT AAA A-3′) and ND6.R3 (5′-CAR GAA TYT ATG TAA AAA CAT TTT G-3′), both also designed for this study. PCR reactions were performed under similar condition to COI gene. The thermocycling profile consisted of one cycle of 2 min at 94°C, five cycles of 1 min at 94°C, 40 s at 37°C and 40 s at 72°C, followed by 45 cycles of 45 s at 94°C, 45 s at 50°C and 1 min at 72°C, with a final extension of 7 min at 72°C. PCR products were electrophoresed in 2% Tris borate-EDTA agarose gels stained with ethidium bromide. PCR products were cycle sequenced in both directions after further cleanup by using polyethylene glycol (PEG) precipitation (20% PEG 8000 and 2.5 N NaCl). The cycle sequencing reaction had a total volume of 10 μl and included 10 pmol of each primer and 1 μl of Big Dye terminator version 3.1. The sequencing reaction protocol consisted of one cycle of 1 min at 96°C followed by 30 cycles of 10 s at 96°C, 5 s at 55°C and 4 min at 60°C. Sequences were analyzed on an ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA). The COI and ND6 sequences were translated into amino acids by using the *Drosophila* genetic code implemented in MacClade 4.0 (Maddison and Maddison 2000) and rechecked to ensure that there were no frame shifts. The sequences have been deposited in GenBank (COI, accession nos. DQ897936–DQ897972; ND6, accession nos. DQ899796–DQ899832).

Phylogenetic Analysis. Unweighted parsimony was performed in PAUP (Swofford 2004) by using a heuristic search with tree-bisection-reconnection (TBR) and 1,000 random-taxon additions. Parsimony bootstrap support values were generated from 1,000 pseudoreplicates with 10 random-taxon-addition replicates per pseudoreplicate. Parsimony uninformative characters were excluded from all the analyses.

Table 1. Location, taxon ID, date, collectors and sex of the samples of the species of the Leucosphyrus Group included in the study

Species	Taxon ID	Date	Collector	Geographical coordinates	Sex	Country	Location
*An. latens*2	IDK43-10	18-23-IX-1986	R. Harbach and V. Baimai	3° 49′ S 115° ′ E	M	Indonesia	Kalimantan, Tana Laut, Salaman
*An. latens*4	PB53L-100	12-XII-1986	V. Baimai	6° 4′ N 100° 1′ E	F	Thailand	Songkhala, Sadao, Padang Besa, Khao Rup Chang
*An. leucosphyrus*1	ID1-002-13	7-IV-1986	R. Harbach and V. Baimai	1° 2′ S 102° ′ E	M	Indonesia	Sumatra Island, Propinsi Jambi, Bukit Baru (near Muarabungo)
*An. leucosphyrus*2	ID1-007-11	7-IV-1986	R. Harbach and V. Baimai	1° 2′ S 102° ′ E	M	Indonesia	Sumatra Island, Propinsi Jambi, Bukit Baru (near Muarabungo)
*An. balabacensis*1	M47	21-XI-1996	R. Harbach	5° 1′ S 117° 45′ E	F	Malaysia	Sabah, Lahad Datu District, Lahad Datu, Borneo rainforest
*An. balabacensis*2	Coll.#53	1989	M. Bangs	3° 51′ S 115° 13′ E	M	Indonesia	south Kalimantan, Salaman, Kintap, kilometer 18
*An. balabacensis*3	M47-16	23-XI-1996	R. Harbach	5° 1′ S 117° 45′ E	F	Malaysia	Sabah, Lahad Datu District, Lahad Datu, Borneo rainforest
*An. dirus*3	09147(29)-3	4-VIII-1982	AFRIMS	14° 16′ N 101° 54′ E	M	Thailand	Prachinburi, Ban Bu Phram
*An. dirus*4	TH1746(6)-17	4-X-1989	V. Baimai	16° 40′ N 98°40′ E	M	Thailand	Tak, Mae Sot, Thum Rua
*An. dirus*5	B(12)1				M	Thailand	Thailand, Bangkok colony
*An. dirus*6	B(15)-11				M	Thailand	Thailand, Bangkok colony
*An. cracens*1	MH0016(1)-4	28-IV-1982	V. Baimai and R. G. Andre	5° 4′ N 102° 56′ E	M	Malaysia	Terengganu, Kampong Dura
*An. cracens*2	MH0023(2)-6	29-IV-1982	V. Baimai and R. G. Andre	5° 6′ N 102° 55′ E	M	Malaysia	Terengganu, Kampong Tapah
*An. scanloni*2	Gass26(5)-21	1987	V. Baimai	14° 25′ N 99° 17′ E	M	Thailand	Kanchanaburi, Sai Yok, Phu Toei
*An. scanloni*4	9120-ISO(5)-8	1-VII-1981	AFRIMS	14° 27′ N 99° 5′ E	M	Thailand	Kanchanaburi, Tha Kradan, Ban Phu Taka, Mu 3
*An. scanloni*5	8-31	1981			M	Thailand	Kanchanaburi Colony
*An. baimaii*2	TH1690(10)-10	7-VIII-1989	V. Baimai	16° 40′ N 98°40′ E	M	Thailand	Mae Sot, Ban Kariang, Thum Rua
*An. baimaii*3	08623(2)-109	26-VI-1982	AFRIMS	8° 17′ N 98° 23′ E	M	Thailand	Phangnga, Khao Pak Chaung (Chong)
*An. baimaii*4	TH504(2)-14	23-II-1986	V. Baimai	8° 35′ N 98° 32′ E	M	Thailand	Phangnga, Ban Bang Kaeo
*An. baimaii*5	TKK-A	1987	M. M.Thu and Myo-Paing	17° 23′ N 96° 3′ E	M	Myanmar	Pegu Division, Taikkyi, 50 miles, north of Yangon
*An. baimaii*6	636-1-L	2-XI-1975	R. Rosenberg	24° 1′N 91° 24′ E	M	Bangladesh	Chaklapungee, Forest Beat
*An. elegans*1	F13(6)-27	11-14-VIII-1981	H. Bhat	14° 19′ N 75° 7′ E, 14° 13′ N 75° 1′ E	M	India	Karnataka, Shimoga, Kondagalale and Shimoga, Keladi
*An. elegans*3	F13(9)-25	11-14-VIII-1981	H. Bhat	14° 19′ N 75° 7′ E, 14° 13′ N 75° 1′ E	M	India	Karnataka, Shimoga, Kondagalale and Shimoga, Keladi
*An. nemophilous*1	08165-11	26-V-1980	AFRIMS	8° 36′ N 98° 32′ E	M	Thailand	Phangnga, Ban Bang Ra Ko
*An. nemophilous*3B	08117(1)-100	16-XI-1979	AFRIMS	14° 25′ N 98° 53′ E	M	Thailand	Kanchanaburi, Huey Sai Yok
*An. nemophilous*4	TH498-100	28-V-1987	AFRIMS	8° 35′ N 98° 32′ E	M	Thailand	Phangnga, Ban Bang Kaeo
*An. takasagoensis*1	F118(6)-8	28-IV-1980	J. C. Lein/AFRIMS	23° 00′ N 120° 00′ E	M	China	Peiyuan, Tungho, Taiwan colony, subcolony Bangkok
*An. takasagoensis*2	F118(4)-16	28-IV-1980	J. C. Lein/AFRIMS	23° 00′ N 120° 00′ E	M	China	Peiyuan, Tungho, Taiwan colony, subcolony Bangkok
*An. takasagoensis*3	F118(3)-1	28-IV-1980	J. C. Lein/AFRIMS	23° 00′ N 120° 00′ E	M	China	Peiyuan, Tungho, Taiwan colony, subcolony Bangkok
*An. mirans*1	424-101/ACC510	7-VIII-1975	E. L. Peyton and Y.-M. Huang	6° 50′ N 80°10′ E	M	Sri Lanka	Western, Colombo, Labugama Reservoir
*An. mirans*3	669-100	22-XII-1977		11° 25′ N 76° 30′ E	M	India	Madras [Tamil Nadu], Nilgiris, Buliar
An. sulawesi	0016-16	23-IX-1985	J. Hii	0° 35′ S 123° 54′ E	M	Indonesia	Toraut, Bone-Dumoga Forest Reserve
*An. macarthuri*1	SL47-117	22-III-1965	AFRIMS	6° 54′ N 100° 15′ E	M	Thailand	Songkhla, Ton Nga Chang Waterfall
*An. macarthuri*2	08161-24	25-V-1980	AFRIMS	8° 35′ N 98° 32′ E	M	Thailand	Phangnga, Ban Bang Kaeo
*An. macarthuri*3	TH485-100/Acc1269	26-V-1987	AFRIMS	8° 38′ N 98° 32′ E	M	Thailand	Phangnga, Ban Bang Ra Ko
*An. macarthuri*5	RN045-104	2004	AFRIMS	Not specified	F	Thailand	Thailand
*An. macarthuri*6	CP001-100	2004	AFRIMS	Not specified	F	Thailand	Thailand

Modeltest version 3.6 (Posada and Crandall 1998) was used to choose a model using the Akaike Information Criterion (AIC). This is similar to the model choice strategy used in Nylander et al. (2004). Consequently, maximum likelihood topology was constructed under the HKYIG model by using PHYML version 2.4.4 (Guindon and Gascuel 2003). Support for each clade generated for unpartitioned data sets was assessed by 100 bootstrap replicates by using PHYML version 2.4.4.

MrBayes version 3.0B4 (Huelsenbeck and Ronquist 2001) was used for Bayesian phylogenetic analyses of the partitioned (ND6 position 1, ND6 position 2, ND6 position 3, COI position 1, and COI position 3) data set without COI position 2. Modeltest version 3.6 was used to choose a model for each partition separately. MrBayes version 3.0B4 does not implement all models suggested by Modeltest; therefore when a subset of the GTR model was suggested by Modeltest then the GTR model was used in MrBayes, with the same among-site rate variation modeling.

As outgroups, we used sequences from GenBank (COI: *An. aquasalis* AF417697, *An. albimanus* AF417695, *An. gambiae* L20934, and *An. quadrimaculatus*A NC_000875) and (ND6: *An. aquasalis* U35260, *An. albimanus* U35259, *An. gambiae* L20934, and *An. quadrimaculatus*A NC_000875).

Results

Sequences of 599 bp (250 bp for COI and 349 bp for ND6) from 41 individuals (four outgroup, 37 ingroup) were obtained from the mitochondrial COI and ND6 genes of 13 ingroup and four outgroup taxa. Individuals with identical sequence were combined and renamed to give 32 unique sequences. Identical sequences are represented on Figs. 1 and 2 as follows: *An. leucosphyrus*1, *An. leucosphyrus*2 is "*leucosphyrus*1_2"; *An. dirus*4, *An. dirus*5, *An. dirus*6, *An. baimaii*3, *An. baimaii* four is "*dirus*4_5_6_*baimaii*3_4"; *An. elegans*1, *An. elegans*3 is "*elegans*1_3"; *An. takasagoensis*1, *An. takasagoensis*2 is "*takasagoensis*1_2"; and *An. macarthuri*3, *An. macarthuri*5, *An. macarthuri*6 is "*macarthuri*3_5_6." Consequently, we analyzed 32 sequences of 13 ingroup and four outgroup taxa. The number of sites, constant and variable sites, and parsimony informative sites are listed in Table 2. Because COI codon position 2 has only two variable sites, it was excluded from all analyses.

The best model for ML and Bayesian analyses was chosen with the aid of Modeltest, which suggested the HKYIG model. Modeltest does not test site-specific models for partitioned data, so to choose how to best model the among-site rate variation (ASRV), the tree used by Modeltest was reevaluated by maximum likelihood (ML) using PAUP with the HKY model and various partitioning and ASRV schemes (Table 2). The IG ASRV on unpartitioned data suggested by Modeltest was much better than no ASRV, and better than a site-specific model based on partitioning the data by gene. However, a site-specific model based on partitioning the data into five codon positions (no COI position 2) had a better likelihood than the unpartitioned IG model, and so this site-specific model with five partitions was used in Bayesian analysis. Because the site-specific model is not implemented in PHYML (and only a simple version is implemented in PAUP), the IG model of ASRV was used in PHYML.

All maximum parsimony (MP) (data not shown), ML (Fig. 1) and Bayesian (Fig. 2) trees based on combined COI and ND6 sequences show nearly identical topologies except for a disagreement in the position of *An. balabacensis*, which arises either as a separate monophyletic group within a major clade formed by members of the Leucosphyrus Group (Fig. 1) or in the Hackeri/Riparis clade (Fig. 2). The ML topology recovered using PHYML version 2.4.4 (Fig. 1) corroborates the monophyly of the Leucosphyrus Group and recovered five subclades. The first subclade [$L_{(a)}$] includes *An. leucosphyrus* and *An. latens*, both members of the Leucosphyrus Complex, the second subclade (H) includes *An. sulawesi* and *An. mirans*, which belong to the Hackeri Subgroup, a third subclade (R) includes *An. macarthuri* of the Riparis Subgroup, a fourth separate subclade [$L_{(b)}$] formed by *An. balabacensis* of the Leucosphyrus Complex and a fifth subclade (D) leading to members of the Dirus Complex (*An. dirus*, *An. baimaii*, *An. elegans*, *An. takasagoensis*, *An. cracens*, *An. scanloni*, and *An. nemophilous*). *An. dirus* and *An. baimaii* clustered together in a clade that is sister to *An. elegans*. Monophyly of the $L_{(a)}$ is ambiguous because *An. latens* and *An. leucosphyrus* sequences clustered together in a poorly supported clade (68% ML bootstrap value) (Fig. 1). In all other analyses the relationship between *An. leucosphyrus* and *An. latens* is unresolved (Fig. 2).

In contrast, the Dirus Complex was recovered as a monophyletic group in all analyses. Relationships among its members were not entirely resolved and varied according to the method used for the analyses. *An. cracens*, *An. takasagoensis*, *An. elegans*, *An. baimaii*, and *An. dirus* were recovered monophyletic. Contrasting, the three sequences of *An. scanloni* did not cluster together in any of the analyses, whereas those of *An. nemophilous* clustered together in a clade within the Dirus Complex. *Anopheles elegans* was always placed as sister to (*An. dirus* and *An. baimaii*) lineage, whereas *An. takasagoensis* was recovered as sister to the (*An. elegans*, *An. dirus*, and *An. baimaii*) subclade. The phylogenetic position of *An. cracens* is not well

Table 2. Description of the Leucosphyrus Group and ND6 and COI sequences used in the analysis

Partition	Sites	Constant	Variable	Parsimony informative
ND6pos1	116	80	36	25
ND6pos2	116	96	20	13
ND6pos3	117	45	72	51
COIpos1	84	74	10	6
COIpos2	83	81	2	0
COIpos2	83	20	63	44

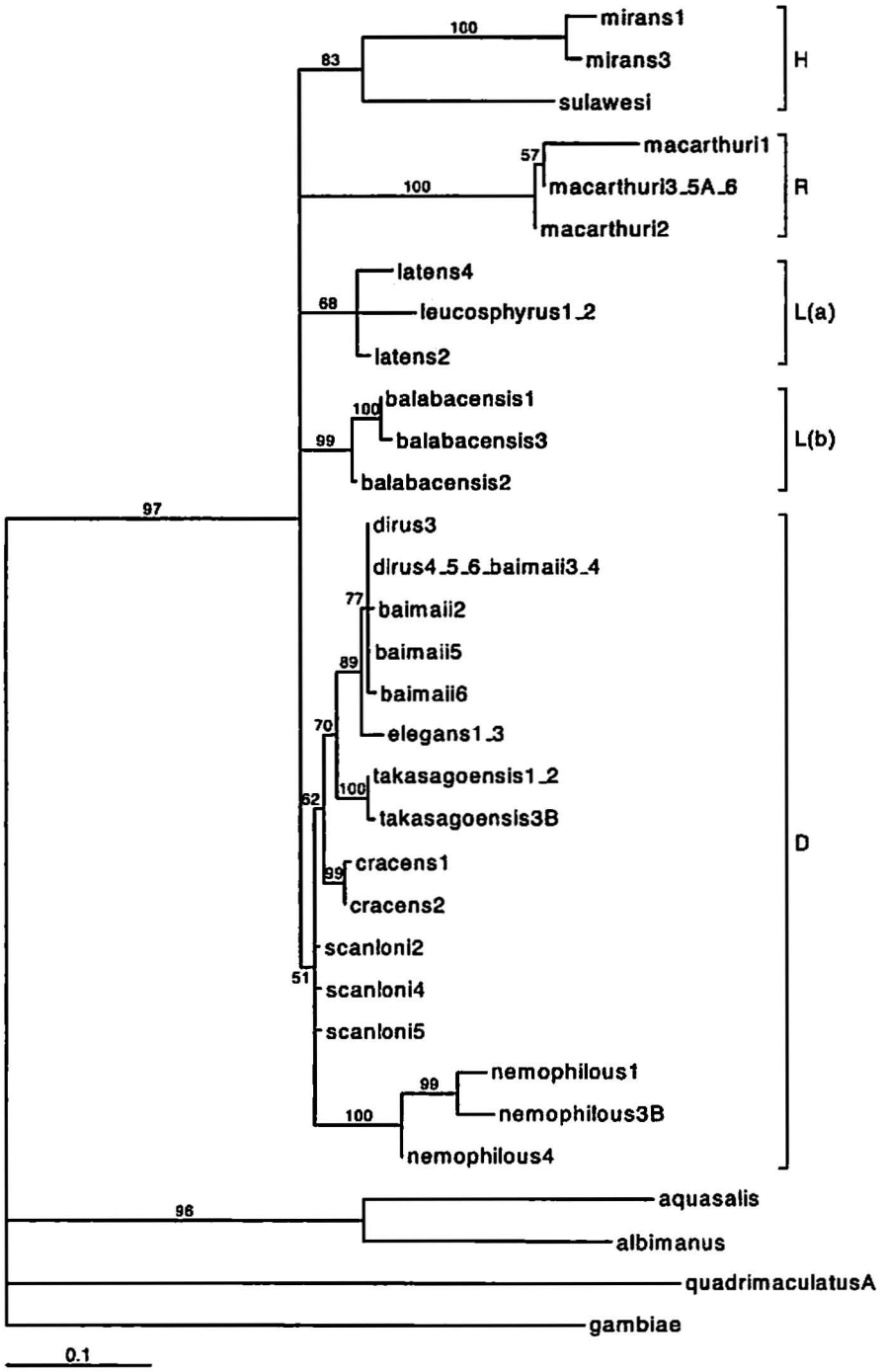

Fig. 1. Maximum likelihood bootstrap topology using the PHYML version 2.4.4, with the HKYIG model. The site specific (SS) model is not implemented in this program.

supported because this species was placed either as sister to (*An. takasagoensis*, *An. elegans*, *An. dirus*, and *An. baimaii*) in both ML and Bayesian topologies (Figs. 1 and 2) or within a polytomy in the Dirus Complex plus the *An. balabacensis* lineage, in the MP strict consensus tree (data not shown).

The hypothesis for monophyly of the Leucosphyrus Group is in ML (97%) and Bayesian (1.0) analyses and moderately well supported in MP analysis (89%). Monophyly of the Leucosphyrus Complex is not supported by any of the analyses; thus, the complex seems to be polyphyletic because it includes *An. balabacen-*

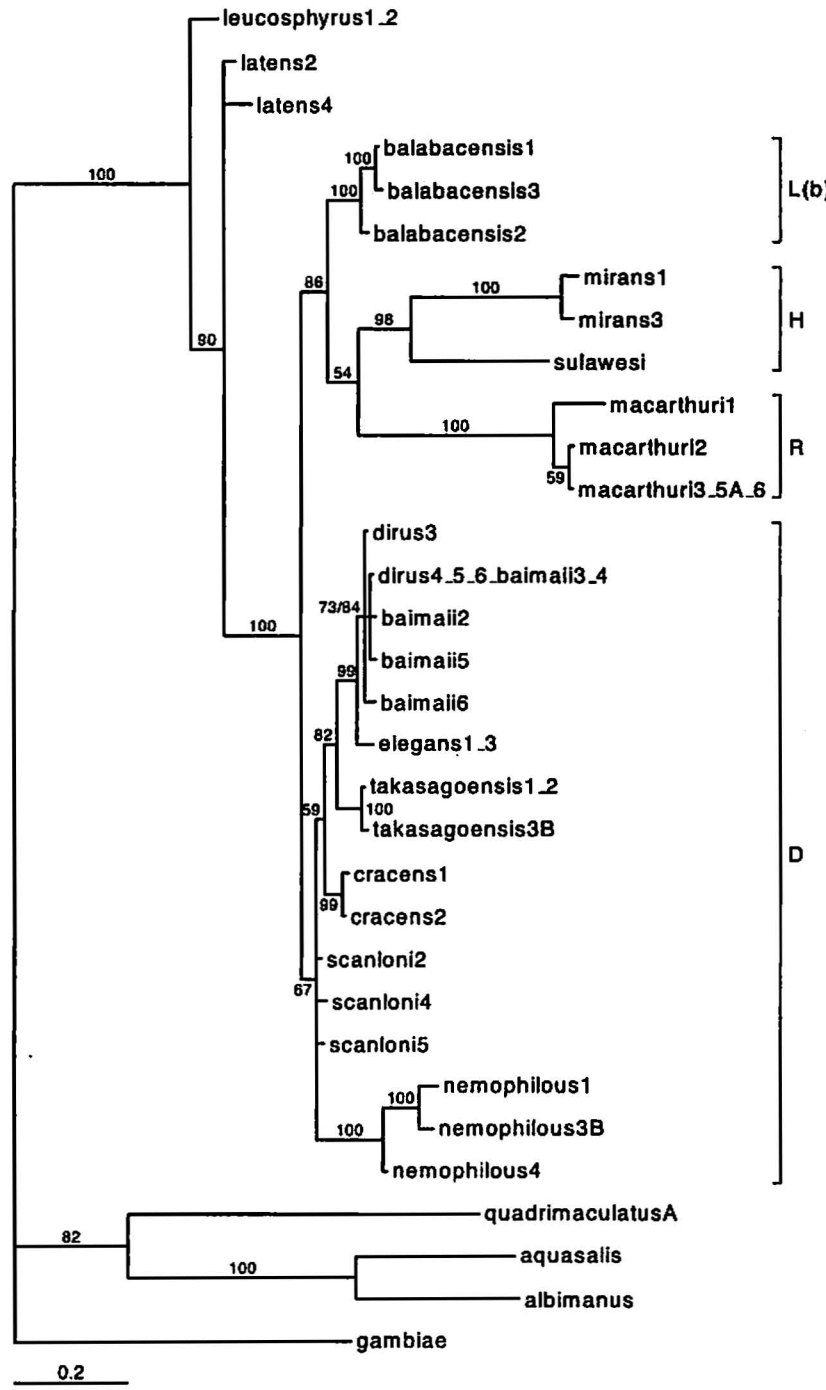

Fig. 2. Bayesian topology generated using MrBayes 3.0B4. The data were divided into five partitions, based on codon position (ND6 position 1, ND6 position 2, ND6 position 3, COI position 1 and COI position 3). The model given to each partition is GTRI; HKYG; GTRG; GTRI and GTRC, respectively. All parameters were "unlinked" in the different partitions. A repeat of this analysis was made, and the consensus tree differed only in that *elegans*1_3 and *dirus*3 reversed positions.

sis. Also, the low bootstrap values do not support a paraphyletic hypothesis for the Dirus Complex, and the phylogenetic position of *An. balabacensis* remains unresolved either as sister to the Dirus Complex or as outgroup of the (Hackeri and Riparis) clade.

Discussion

COI mitochondrial DNA sequences have been used for studying genetic population structure of species of the *An. dirus* complex. Consequently, an almost complete absence of mtDNA differentiation between *An. dirus* and *An. baimaii* could possibly suggest either mtDNA historical introgression between these species or a selective sweep that originated in *An. baimaii* (Walton et al. 2000). Additionally, Walton et al. (1999) identified an *An. scanloni-An. baimaii* hybrid among field-collected specimens, showing that there is a potential for introgression between this species pair. Jiggins (2004) investigated the association between mitochondria and male-killer *Wolbachia* in two species of butterflies of the genus *Acraea* and showed that these parasites can reduce intraspecific polymorphism and cause interspecific introgression of mtDNA. Hypothetically, a cause but *Wolbachia* infection has not yet been observed in Southeast Asian *Anopheles* (Kittayapong et al. 2000). In agreement with Walton et al. (2000), results of the current study found identical ND6 and COI sequences for both *An. dirus* and *An. baimaii*, but there is no evidence for introgression in any other species. Additionally, there seems to be very low intraspecific variation in both genes, and thus we found identical sequences for *An. leucosphyrus*, *An. elegans*, *An. takasagoensis*, and *An. macarthuri*, whereas except for *An. dirus* and *An. baimaii* interspecific variation seems to be higher.

In a combined analysis of the COI and ND6 gene regions, the traditionally recognized Leucosphyrus Group was found to be a strongly supported monophyletic assemblage within the subgenus *Cellia*. However, our results revealed that the current classification of Leucosphyrus Subgroup is composed of unnatural assemblages. In none of the topologies recovered using different methods of phylogenetic analysis were the Dirus and Leucosphyrus Complexes recovered as sisters. We also provide evidence that the Leucosphyrus Complex is not monophyletic because *An. balabacensis* did not cluster with the two other species of the subgroup included in our study, *An. latens* and *An. leucosphyrus*. It is noteworthy that *An. balabacensis* was recovered either in the clade leading to the Hackeri and Riparis Subgroups (Fig. 2) or as a separate lineage within the Leucosphyrus Group (Fig. 1). The Dirus Complex is a monophyletic lineage. Also, the Riparis and Hackeri Subgroups were recovered as sister groups (Fig. 2) or as a polytomy in the Leucosphyrus Group. Relationships between *An. balabacensis* and the Riparis and the Hackeri Subgroups are not supported by a morphological hypothesis. According to Sallum et al. (2005b) the morphological distinction between the Leucosphyrus and the Dirus Complexes is problematic because some characters used to define the limits of each species complex are polymorphic. Generally, members of the Leucosphyrus Complex can be distinguished easily from those of the Dirus Complex in having the accessory sector pale (ASP) wing spot present on veins C, subcosta and R, and by the absence of pale scales at the base of hindtarsomere 4. However, *An. balabacensis* is polymorphic for these characters and thus can overlap with members of both the Dirus Complex and the Leucosphyrus Complex. A sister group relationship between *An. balabacensis* and members of the Hackeri and Riparis Subgroups has no morphological support and *An. balabacensis* can be separated easily from members of the two subgroups (Sallum et al. 2005b). The placement of *An. balabacensis* as sister to the Dirus Complex is more concordant with a morphological hypothesis than as sister to the (Riparis and Hackeri) clade. Additionally, Kanda et al. (1983) compared seven populations of members of the Leucosphyrus Group and showed that *An. balabacensis* was not distinct from *An. dirus*. Similarly, Yong et al. (1983) used 15 gene–enzyme systems to compare genetic diversity in *An. dirus*, *An. cracens*, and *An. balabacensis* and found that the three taxa were monomorphic for all 15 loci tested. Consequently, it is obvious that *An. balabacensis* is genetically closely related to members of the Dirus Complex. It is also possible that *An. balabacensis* represents a widespread species complex in Southeast Asia.

Although our results support the monophyly of the Dirus Complex, relationships among its members are only moderately to weakly supported (Figs. 1 and 2). Generally, within the complex, we can recognize two major groups, one group poorly supported group consisting of *An. nemophilous* and *An. scanloni* (data not shown) and a second group leading to (*An. cracens* (*An. takasagoensis* (*An. elegans* (*An. baimaii*, *An. dirus*)))). Sequences of *An. scanloni*2, *An. scanloni*4, and *An. scanloni*5 did not cluster together in any of the analyses by using several methods. In the Bayesian analysis, *An. scanloni*4 clustered within the *An. nemophilous* clade, whereas *An. scanloni*2 and *An. scanloni*5 formed a subclade that is sister to *An. nemophilous* plus *An. scanloni*4 (data not shown). Walton et al. (2000, 2001) demonstrated *An. scanloni* to be a well-differentiated species and that the high degree of differentiation between northern and southern populations of *An. scanloni* was suggestive of the presence of two incipient species. Our results also suggest that there might be at least two subpopulations within *An. scanloni* because *An. scanloni*4 clustered with *An. nemophilous*, whereas *An. scanloni*2 and *An. scanloni*5 either clustered together (data not shown) or were recovered in a polytomy within the Dirus Complex (Figs. 1 and 2). Placement of *An. elegans* in the clade consisting of (*An. dirus*, *An. baimaii*) might support the hypothesis of Sawadipanich et al. (1990) that there are cytogenetic and crossing evidence that *An. elegans* is an incipient sibling species of *An. baimaii*.

Results of the current study and other studies on genetic relationships among members of the Dirus Complex do not always coincide. For example, Manguin et al. (2002) showed that *An. dirus* and *An. scanloni* are closer to each other than either is to *An. cracens* or *An. baimaii* because they share an 888-bp SCAR fragment. Similarly, results of laboratory crossing experiments and polytene and mitotic chromosome (Baimai et al. 1987) are consistent in demon-

strating *An. dirus* and *An. scanloni* to be closely related, and that *An. dirus* and *An. baimaii* were genetically the most incompatible in comparison with the other species tested. Moreover, Poopittayasataporn and Baimai (1995) suggested that *An. baimaii* may be the most basal species within the Dirus Complex.

Results of the current study do not fully resolve relationships within the Leucosphyrus Group. They failed to show monophyly of the Leucosphyrus Subgroup and the phylogenetic placement of *An. balabacensis*. However, this study corroborates the monophyly of the Leucophyrus Group and the Dirus Complex and shows an initial indication of the monophyly of the Hackeri and Riparis Subgroups. A more extensive sampling of species within the Leucosphyrus Group will be critical to test the monophyly of the Leucosphyrus, Hackeri and Riparis subgroups and to establish the phylogenetic position of *An. balabacensis* and the two poorly known Con Son and Negros Forms. In addition, it will provide a stronger basis for future biogeographical studies and co-evolutionary studies of *Anopheles* species in relation to simian and human malaria.

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References Cited

Baimai, V., and C. A. Green. 1985. Cytogenetic studies of *Anopheles balabacensis* and *Anopheles maculatus* complexes in Thailand. Southeast Asian J. Trop. Med. Public Health 16: 187.

Baimai, V., B. A. Harrison, and V. Nakavachara. 1980. The salivary gland chromosomes of *Anopheles* (*Cellia*) *dirus* (Diptera: Culicidae) of the Southeast Asian Leucosphyrus Group. Proc. Entomol. Soc. Wash. 82: 319–328.

Baimai, V., R. G. Andre, and B. A. Harrison. 1984a. Heterochromatin variation in the sex chromosomes in Thailand populations of *Anopheles dirus* A (Diptera: Culicidae). Can. J. Genet. Cytol. 26: 633–636.

Baimai, V., C. A. Green, R. G. Andre, B. A. Harrison, and E. L. Peyton. 1984b. Cytogenetic studies of some species complexes of *Anopheles* in Thailand and Southeast Asia. Southeast Asian J. Trop. Med. Public Health 15: 536–546.

Baimai, V., R. G. Andre, B. A. Harrison, U. Kijchalao, and L. Panthusiri. 1987. Crossing and chromosomal evidence for two additional sibling species within the taxon *Anopheles dirus* Peyton and Harrison (Diptera: Culicidae) in Thailand. Proc. Entomol. Soc. Wash. 89: 157–166.

Baimai, V., R. E. Harbach, and U. Kijchalao. 1988a. Cytogenetic evidence for a fifth species within the taxon *Anopheles dirus* in Thailand. J. Am. Mosq. Control Assoc. 4: 333–338.

Baimai, V., R. E. Harbach, and S. Sukowati. 1988b. Cytogenetic evidence for two species within the current concept of the malaria vector *Anopheles leucosphyrus* in Southeast Asia. J. Am. Mosq. Control Assoc. 4: 44–50.

Baimai, V., A. Poopittayasataporn, and U. Kijchalao. 1988c. Cytological differences and chromosomal rearrangments in four members of the *Anopheles dirus* complex (Diptera: Culicidae). Genome 30: 372–379.

Barcus, M. J., F. Laihad, M. Sururi, P. Sismadi, H. Marwoto, M. J. Bangs, and J. K. Baird. 2002. Epidemic malaria in the Menoreh Hills of central Java. Am. J. Trop. Med. Hyg. 66: 287–292.

Coatney, G. R., W. E. Collins, M. Warren, and P. G. Contacos. 1971. The primate malarias. National Institute of Health, U.S. Department of Health, Education and Welfare, Bethesda, MD.

Colless, D. H. 1956. The *Anopheles leucosphyrus* group. Trans. R. Entomol. Soc. Lond. 108: 37–116.

Damrongphol, P., and V. Baimai. 1989. Scanning electron microscope observations and differentiation of eggs of the *Anopheles dirus* complex. J. Am. Mosq. Control Assoc. 5: 563–568.

Deng, D., H.-L. Qian, and X.-S. Yang. 1982. Rectification of Hainan's *Anopheles b. balabacensis* to *A. dirus* Peyton et Harrison, 1979. Acta Zootaxonomica Sin. 7: 332–333.

Dutta, P., D. R. Bhattacharyya, and L. P. Dutta. 1991. Epidemiological observations on malaria in some parts of Tengakhat PHC Dibrugarh District Assam. Indian J. Malariol. 28: 121–128.

Eyles, D. E., R. H. Wharton, W. H. Cheong, and M. Warren. 1964. Studies on malaria and *Anopheles balabacensis* in Cambodia. Bull. World Health Organ. 30: 7–21.

Fooden, J. 1994. Malaria in macaques. Int. J. Primatol. 15: 573–596.

Green, C. A., L. E. Munstermann, S. G. Tan, S. Panyim, V. Baimai. 1992. Population genetic evidence for species A, B, C and D of the *Anopheles dirus* complex in Thailand and enzyme electromorphs for their identification. Med. Vet. Entomol. 6: 29–36.

Guindon, S., and O. Gascuel. 2003. PHYML–a simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52: 696–704.

Harbach, R. E. 2004. The classification of the genus *Anopheles* (Diptera: Culicidae): a working hypothesis of phylogenetic relationships. Bull. Entomol. Res. 94: 537–553.

Huelsenbeck, J. P., and F. Ronquist. 2001. MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17: 754–755.

Huong, N. T., P. Sonthayanon, A. J. Ketterman, and S. Panyim. 2001. A rapid polymerase chain reaction based method for identification of the *Anopheles dirus* sibling species. Southeast Asian J. Trop. Med. Public Health 32: 615–620.

Ismail, I.A.H., N. Notananda, and J. Schepens. 1974. Studies on malaria and responses of *Anopheles balabacensis balabacensis* and *Anopheles minimus* to DDT residual spraying in Thailand. Part I. Pre-spraying observations. Acta Trop. 31: 129–164.

Ismail, I.A.H., N. Notananda, and J. Schepens. 1975. Studies on malaria and responses of *Anopheles balabacensis balabacensis* and *Anopheles minimus* to DDT residual spraying in Thailand. Part II. Post-spraying observations. Acta Trop. 32: 206–231.

Jiggins, F. M. 2004. Male-killing *Wolbachia* and mitocondrial DNA: selective sweeps, hybrid introgression and parasite population dynamics. Genetics 164: 3–12.

Kanda, T., K. Takai, G. L. Chiang, K. P. Loong, S. Sucharit, and W. H. Cheong. 1983. Phylogenetic interpretation and chromosomal polymorphism among nine strains of human malaria vectors of the *Anopheles leucosphyrus* group. Jpn. J. Genet. 58: 193–208.

Kittayapong, P., K. J. Baisley, V. Baimai, S. L. O'Neill. 2000. Distribution and diversity of *Wolbachia* infections in Southeast Asian mosquitoes (Diptera: Culicidae). J. Med. Entomol. 37: 340–345.

Maddison, D. R., and M. D. Maddison. 2000. MacClade 4. Analysis of phylogeny and character evolution. Sinauer, Sunderland, MA.

Manguin, S., P. Kengne, L. Sonnier, R. E. Harbach, V. Baimai, H. D. Trung, and M. Coosemans. 2002. SCAR markers and multiplex PCR-based identification of isomorphic species in the *Anopheles dirus* complex in Southeast Asia. Med. Vet. Entomol. 16: 46–54.

Nylander, J.A.A., F. K. Ronquist, J. P. Huelsenbeck, and J. L. Nieves-Aldrey. 2004. Bayesian phylogenetic analysis of combined data. Syst. Biol. 53: 47–67.

Palumbi, S. R. 1996. Nucleic acids II: the polymerase chain reaction, pp. 205–247. *In* D. M. Hillis, C. Moritz, and B. K. Mable [eds.], Molecular systematics. Sinauer, Sunderland, MA.

Peyton, E. L. 1989. A new classification for the Leucosphyrus Group of *Anopheles (Cellia)*. Mosq. Syst. 21: 197–205.

Peyton, E. L., and B. A. Harrison. 1979. *Anopheles (Cellia) dirus*, a new species of the Leucosphyrus Group from Thailand (Diptera: Culicidae). Mosq. Syst. 11: 40–52.

Peyton, E. L., and S. Ramalingam. 1988. *Anopheles (Cellia) nemophilous*, a new species of the Leucosphyrus Group from Peninsular Malaysia and Thailand (Diptera: Culicidae). Mosq. Syst. 20: 272–299.

Poopittayasataporn, A., and V. Baimai. 1995. Polytene chromosome relationships of five species of the *Anopheles dirus* complex in Thailand. Genome 38: 426–434.

Posada, D., and K. A. Crandall. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14: 817–818.

Prakash, A., D. R. Bhattacharyya, P. K. Mohapatra, and J. Mahanta. 2001. Estimation of vectorial capacity of *Anopheles dirus* (Diptera: Culicidae) in a forested-fringed village of Assam (India). Vector Borne Zoonotic Dis. 1: 231–237.

Rahman, M., M. D. Elias, and M. Ameen. 1977. Bionomics of *Anopheles balabacensis balabacensis* Baisas (Diptera: Culicidae) in Bangladesh and its relation to malaria. Bangladesh J. Zool. 5: 1–23.

Reid, J. A. 1968. *Anopheles* mosquitoes of Malaya and Borneo. Stud. Inst. Med. Res. Malaysia 31: 1–520.

Rosenberg, R., and N. P. Maheswary. 1982. Forest malaria in Bangladesh. II. Transmission by *Anopheles dirus*. Am. J. Trop. Med. Hyg. 31: 183–191.

Sallum, M.A.M., T. R. Schultz, and R. C. Wilkerson. 2000. Phylogeny of Anophelinae based on morphological characters. Ann. Entomol. Soc. Am. 93: 745–775.

Sallum, M.A.M., E. L. Peyton, and R. C. Wilkerson. 2005a. Six new species of the *Anopheles leucosphyrus* group, with reinterpretation of *An. elegans* and vector implications. Med. Vet. Entomol. 19: 158–199.

Sallum, M.A.M., E. L. Peyton, B. A. Harrison, and R. C. Wilkerson. 2005b. Revision of the Leucosphyrus Group of *Anopheles (Cellia)* (Diptera: Culicidae). Rev. Bras. Entomol. 49: 1–152.

Sawadipanich, Y., V. Baimai, and B. A. Harrison. 1990. *Anopheles dirus* species E: chromosomal and crossing evidence for another member of the Dirus Complex. J. Am. Mosq. Control Assoc. 6: 477–481.

Scanlon, J. E., and U. Sandhinand. 1965. The distribution and biology of *Anopheles balabacensis* in Thailand (Diptera: Culicidae). J. Med. Entomol. 2: 61–69.

Schultz, G. W. 1992. Biting activity of mosquitos (Diptera: Culicidae) at a malarious site in Palawan, Republic of Philippines. Southeast Asian J. Trop. Med. Public Health 2: 464–469.

Sloof, R., and J. Verdrager. 1972. *Anopheles balabacensis balabacensis* Baisas, 1936 and malaria transmission in south-eastern areas of Asia. WHO/Mal/72.765.

Swofford, D. L. 2004. PAUP*: phylogenetic analysis using parsimony (*and other methods). Sinauer, Sunderland, MA.

Trung, H. D., W. Van Bortel, T. Sochantha, K. Keokenchanh, N. T. Quang, L. D. Cong, and M. Coosemans. 2004. Malaria transmission and major malaria vectors in different geographical areas of Southeast Asia. Trop. Med. Int. Health 9: 230–237.

Tsukamoto, M., A. Miyata, and I. Miyagi. 1978. Surveys on simian malaria parasites and their vector in Palawan Island, the Philippines. Trop. Med. 20: 39–50.

Walton, C., J. M. Handley, C. Kuvangkadilok, F. H. Collins, R. E. Harbach, V. Baimai, and R. K. Butlin. 1999. Identification of five species of the *Anopheles dirus* complex from Thailand, using allele-specific polymerase chain reaction. Med. Vet. Entomol. 13: 24–32.

Walton, C., J. M. Handley, W. Tun-Lin, F. H. Collins, R. E. Harbach, V. Baimai, and R. K. Butlin. 2000. Population structure and population history of *Anopheles dirus* mosquitoes in Southeast Asia. Mol. Biol. Evol. 17: 962–974.

Walton, C., J. M. Handley, F. H. Collins, V. Baimai, R. E. Harbach, V. Deesin, and R. K. Butlin. 2001. Genetic population structure and introgression in *Anopheles dirus* mosquitoes in South-east Asia. Mol. Ecol. 10: 569–580.

Warren, M., and R. H. Wharton. 1963. The vectors of simian malaria: identity, biology, and geographical distribution. J. Parasitol. 49: 892–904.

Warren, M., D. E. Eyles, R. H. Wharton, and C. K. Ow Yang. 1963. The susceptibility of Malayan anophelines to *Plasmodium cynomolgi bastianellli*. Indian J. Malariol. 17: 85–105.

White, G. B. 1983. The importance of *Anopheles leucosphyrus* group mosquitoes as vectors of malaria and filariasis in relation to transmigration and forestry in Indonesia with assessments of *Anopheles balabacensis* ecology and vectorial capacity. Assignment Report: 12 July–15 September 1982. WHO Project IR VBC 025. World Health Organization. Vector Biology and Control Research Unit, Jakarta, Indonesia.

Wilkinson, R. N., D. J. Gould, P. Boonyakanist, and H. E. Segal. 1978. Observations on *Anopheles balabacensis* (Diptera: Culicidae) in Thailand. J. Med. Entomol. 14: 666–671.

Yong, H. S., W. H. Cheong, G. L. Chiang, S. S. Dhaliwal, K. P. Loong, and K. P. Sarjan. 1983. Biochemical genetic relationship in three taxa of the malaria mosquito *Anopheles balabacensis* complex. Comp. Biochem. Physiol. 76: 611–613.

Zulueta, J. 1956. Malaria in Sarawak and Brunei. Bull. World Health Organ. 15: 65–671.

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