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Systematics of *Anopheles barbirostris* van der Wulp and a sibling species of the Barbirostris Complex (Diptera: Culicidae) in eastern Java, Indonesia

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Abstract. This study provides the first integrated morphological and molecular characterization of *Anopheles barbirostris* van der Wulp, the nominotypical member of the Barbirostris Complex of malaria vectors in the Oriental Region, and *An. vanderwulpi* **sp.n.**, a sibling species of the complex found in sympatry with *An. barbirostris* in the vicinity of its type locality in eastern Java, Indonesia. The adult, larval and pupal stages of *An. barbirostris* are described and compared with those of *An. vanderwulpi*. The two species, however, are essentially isomorphic. The genetic identity of *An. barbirostris* s.s. is based on a diagnostic cytochrome oxidase I gene sequence to ensure stable use of the specific name for the prevalent concept of the species. The genetic identity of the new species is also established. Diagnostic DNA sequences for these species serve as a foundation for further taxonomic studies, and for investigations into their roles in the transmission of malaria and filariasis. The discussion includes a brief review of *Anopheles* classification and species complexes.

Introduction

Anopheles (*Anopheles*) *barbirostris* van der Wulp and 12 related species comprise the medically important and taxonomically complex Barbirostris Group of malaria vectors in the Oriental Region. Six of these species, including *An. barbirostris*, comprise a subgroup (Barbirostris Subgroup) of species that are almost identical in adult morphology but differ in their roles in the transmission of malaria and filariasis in Southeast Asia.

Mosquitoes identified traditionally as *An. barbirostris* are common and widely distributed from India through mainland Southeast Asia and southward into Indonesia where they are widely distributed in Sumatra, Java, Bali, Kalimantan, Sulawesi and throughout the Lesser Sunda Islands to Timor, with a very limited presence in the Maluku (Mollucas) Islands (Buru Island) (Reid, 1968; Harrison & Scanlon, 1975; O'Connor & Sopa, 1981). Published records of *An. barbirostris* in the Philippines refer to other species of the Barbirostris Group (Reid, 1962). *Anopheles barbirostris* is generally found in greater abundance in upland areas (Harrison & Scanlon, 1975; Ndoen *et al*., 2010), but in West Timor it is associated closely with coastal areas (Ndoen *et al*., 2010) where it is has been incriminated as a malaria vector. *Anopheles barbirostris* appears to prefer to rest outdoors in western and central Java but indoors in West Timor (Ndoen *et al*., 2011), with a preference to rest in human dwellings in West Timor and animal shelters in Java.

The first evidence that *An. barbirostris* in Indonesia may consist of a complex of species came from the studies of Satoto (2001). Based on sequences of the mitochondrial DNA (mtDNA) cytochrome oxidase I gene (COI), Satoto identified four putative species, informally designated W, X, Y and Z, and distinguished these from *An. barbumbrosus* Strickland &

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Chowdhury. Species W and X were identified from Central Java (Jambu and Salaman) and Flores (Reo in Manggarai Regency and Singaraja in East Flores Regency), whereas species Z was identified from the vicinity of Manado in North Sulawesi Province and Palopo in South Sulawesi Province. Three specimens from Thailand represented a separate clade that was informally denoted as species Y but which a maximum parsimony tree suggests is distant from his species X, W and Z (and most likely was *An. campestris* Reid).

In more recent studies, sequence data for the COI gene and the ITS2 rDNA locus (Saeung *et al*., 2007, 2008; Paredes-Esquivel *et al*., 2009) indicate that *An. barbirostris* is a complex of three to five sibling species with undefined distributions. The question of how many species constitute the Barbirostris Complex needs to be resolved, because their recognition and identification have important implications for the control of malaria and lymphatic filariasis. *Anopheles barbirostris* s.l. is considered an important vector of malaria and Brugian filariasis in Sulawesi, Flores and the Timor islands (Atomosoedjono [*sic*] *et al*., 1976; Atmosoedjono *et al*., 1977; Lien *et al*., 1977; Reid *et al*., 1979; Cooper *et al*., 2010), whereas it appears to be a non-vector in most other regions (Reid, 1962). Both *Plasmodium falciparum* and *Plasmodium vivax* infections have been detected in this species in northern Sulawesi (Meras and Tomohon), Flores (Korowuru and Tilang) and Adonara Island in the Lesser Sundas (Bangs & Rusmiarto, 2007). It is a confirmed vector of falciparum malaria in Sri Lanka (Amerasinghe *et al*., 1999) and Timor-Leste (Cooper *et al*., 2010), based on the enzyme-linked immunosorbent assay (ELISA) detection of sporozoites in the head-thorax portions of infected females, which in the case of the latter study were collected as they landed on humans. Both *P. vivax* and *P. falciparum* have been detected by ELISA in females of *An. barbirostris* s.l. in Bangladesh, but it is not known whether sporozoites or oocysts, or both, were present, as whole mosquitoes were assayed for infections (Alam *et al*., 2010). A recent survey in northern Sumatra (Nias Island) identified *An. barbirostris* s.l. as a potential vector of malaria (Syafruddin *et al*., 2007), and Limrat *et al*. (2001) and Apiwathnasorn *et al*. (2002) reported that either *An. barbirostris* or *An. campestris* (these species could not be reliably distinguished) is a probable vector of malaria in Sa Kaeo Province in eastern Thailand, where high numbers of females were captured landing on humans both indoors and outdoors.

Saeung *et al*. (2007, 2008) and Suwannamit *et al*. (2009) provided evidence for three or four species within *An. barbirostris* based on a series of cross-mating experiments (also Choochote *et al*., 1983), cytogenetic studies and sequence analysis of ITS2, COI and COII using isolines derived from wild-caught females. Unfortunately, a comparison of COI sequence data obtained by Paredes-Esquivel *et al*. (2009) with those from Saeung *et al*. (2008) proved to be impossible because the regions sequenced do not overlap. The A3 form of Saeung *et al*. (2008) has a much smaller ITS2 amplicon than the corresponding region investigated by Paredes-Esquivel *et al*. (2009), suggesting that it is not closely related to *An. barbirostris* s.l. Sequence comparisons showed that clades I and II of Paredes-Esquivel *et al*. (2009) were not included in the analyses of Saeung *et al*. (2008), and that clades III and V of Paredes-Esquivel *et al*. (2009) correspond to form A1 and *An. campestris* of Saeung *et al*. (2008), which they described as zoophilic and more anthropophilic species, respectively. Zoophilic and anthropophilic forms of *An. barbirostris* were previously reported by Lien *et al*. (1977), but these behavioural differences, which would influence their capacity to transmit malaria protozoa or filarial nematodes, could not be associated with distinct morphological characters (Reid *et al*., 1979). Saeung *et al*. (2008) identified specimens with ITS2 sequences similar to clade IV of Paredes-Esquivel *et al*. (2009) as *An. barbirostris*; however, specimens of clade IV are morphologically distinct from *An. barbirostris*. Variation of repeat sequences in the ITS2 region of the five purported species of the Barbirostris Complex in Thailand (Saeung *et al*., 2008; Suwannamit *et al*., 2009) was investigated by Otsuka (2011).

Based on available data, it is not possible to determine which species correspond to malaria vector populations. Further analyses require extensive sampling in areas where *An. barbirostris* has been reported to be anthropophagic, such as Sulawesi (Lien *et al*., 1977) and Flores (Reid *et al*., 1979). The molecular data reported herein show that clades I and II of Paredes-Esquivel *et al*. (2009) occur in the type locality of *An. barbirostris* in eastern Java, but which of these two genetic species is conspecific with *An. barbirostris* s.s. is unknown. Hence, the purpose of this paper is to characterize and fix the identity of *An. barbirostris* and to distinguish and formally name the other species of the Barbirostris Complex that also occurs in the environs of the type locality.

Material and methods

This study is based on specimens of two species of the Barbirostris Complex collected in sympatry in the vicinity of the type locality of *An. barbirostris*.

Morphology

Wild-caught larvae and the progeny of wild-caught females were individually reared to provide adults with associated larval and pupal exuviae. Some adults obtained from wild-caught larvae and some from progeny broods were identified as clades I and II of Paredes-Esquivel *et al.* (2009) by sequencing the COI gene of mtDNA (see below). Unambiguously identified specimens were used for comparative anatomical study. Adults were studied using stereomicroscopy and simulated natural light. Larval and pupal chaetotaxy were studied using differential interference contrast microscopy. The specimens have been deposited in the Natural History Museum (NHM), London. The anatomical terminology follows Harbach & Knight (1980, 1982), as revised and updated by Harbach (2012). The symbols ♀, ♂, Le, Pe, L, P and E used in the synonymies, literature summaries and material examined sections represent

female(s), male(s), larval exuviae, pupal exuviae, fourth-instar larvae, pupae and egg(s), respectively. An asterisk (*) following these symbols indicates that at least part of the life stage is illustrated in the corresponding publication.

DNA sequences

DNA was extracted from individual dried specimens either mounted on pins or stored in Beem® capsules over silica gel, using the methodology of Ballinger-Crabtree *et al.* (1992), with an additional series of phenol/chloroform/isoamyl alcohol extraction steps as described in Townson *et al*. (1999), to remove contaminants that may impede PCR amplification.

Cytochrome oxidase I

An 877 bp fragment within the mtDNA COI locus was amplified using the primers and thermal profile described in Paredes-Esquivel *et al.* (2009), with 35 amplification cycles. Reaction mixes of 25 μL contained 1X Kapa Taq buffer A, 0.8 mM dNTPs, 3 mM $MgCl_2$, 0.5 μM of each primer, 0.4 units of Kapa Taq and 2 μL of genomic DNA. PCR products were purified using Bioline SureClean reagent (Bioline Reagents Ltd, U.K.) according to the manufacturer's instructions, and sequenced at Macrogen Korea using an ABI3730XL sequencer (Applied Biosystems, CA, U.S.A.).

DNA extracted from two specimens (JV40-17 and JV18-11, see below) was insufficient to provide full-length COI products; hence, primers were designed to amplify shorter fragments. The primers used were: forward primer COI.F as in Paredes-Esquivel *et al*. (2009) with reverse primer COI-1068R (CCYCCHACTGTAAATAAAAATACAAA) used to produce a 351 bp product and forward primer COI-1993.F (TTGC YGTTCCTACWGGAATTA) with reverse primer COI-1348R (GGAAAATCWGARTATCGTCGAG) to produce a 397 bp product. In both of these reactions, the thermal profile was 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 30 s. In addition, forward primer COI-1335F (CCWCAACAYTTTTTAGGAT), with reverse primer CUL.R as in Paredes-Esquivel *et al*. (2009), was used to produce a 304 bp product. In this reaction, the thermal profile was 94°C for 30 s, 50°C for 30 s and 72°C for 30 s. For all of these, the reaction mixes, purification and sequencing were the same as indicated above. Accession numbers for the sequences of JV40-17 and JV18-11 are listed with the 'Type series' of *An. vanderwulpi* **sp.n.** (see below), but the sequences were not included in the phylogenetic tree (see below) because they contain some missing data. When the COI sequences for JV40-17 and JV18-11 were included in the trees, they fell into clade II (not shown).

Internal transcribed spacer 2

Within the Barbirostris Subgroup, the ITS2 amplicon is exceptionally large (>1.5 kb), larger than in any other *Anopheles* sp., due to a complex series of internal repeats (Paredes-Esquivel *et al.*, 2009; Otsuka, 2011). Consequently, sequencing this region involved amplification followed by cloning, as described in Paredes-Esquivel *et al*. (2009). For the present study, ITS2 amplification was carried out solely to determine the size of the ITS2 amplicon as an additional means of separating the two species within the type locality in eastern Java. ITS2 was amplified using 5.8S forward and 28S reverse primers and the thermal profile described in Paredes-Esquivel *et al.* (2009), with 35 amplification cycles. Reaction mixes, purification and sequencing were as described earlier in this paper. Products of ITS2 amplification were separated on 0.8% agarose gels, yielding a dominant product of 1545 bp for clade I and 1727 bp for clade II. The size of the ITS2 amplicon in other clades cited by Paredes-Esquivel *et al.* (2009) were as follows: clade III, 1730 bp; clade IV, 1583 bp; and clade V, 1519 bp. Hence, the estimated length of the ITS2 amplicon is insufficient to separate all clades, whereas the COI sequence provides an unequivocal attribution of specimens to clades.

Phylogenetics

Sequence traces generated from each forward and reverse primer were examined and aligned using CODONCODE ALIGNER version 3.7.1 (Codon-Code Corporation, Dedham, MA, U.S.A.). FASTA sequences for each individual were imported into MEGA version 5 (Tamura *et al*., 2011) for alignment and trimming. For phylogenetic analysis, the following sequences from Paredes-Esquivel *et al*. (2009) were added to the alignment: *An. barbirostris* clade I from South Kalimantan, Indonesia (EU797194–EU797197) and Thailand (EU797198–EU797203), *An. barbirostris* clade II (EU797204), *An. barbirostris* clade III (EU797230–EU797231) and clade IV (EU797273–EU797274), which is morphologically distinct from *An. barbirostris*. *Anopheles coustani* Laveran (AF417715A) and *An. campestris* (EU797277) were used as the outgroup.

Unique haplotypes were identified, and a haplotype network with a connection limit of 95% probability of parsimony was calculated using TCS 1.2.1 (Clement *et al*., 2000) (network not shown). Several of the new sequences were found to be identical to *An. barbirostris* clade II (EU797204).

A Bayesian phylogeny was estimated using MRBAYES version 3.1.2 (Ronquist & Huelsenbeck, 2003) with six substitution rates, and a proportion of invariable sites. Two runs of four chains were run for 1 000 000 generations, with samples taken once every 100 generations. The first 1000 trees (1×10^5 generations) were discarded as burn-in, and a consensus tree was produced from the remaining 9001 samples. The analysis was run twice, giving identical tree topologies and very similar posterior probabilities.

PHYML version 3.0 (Montpellier Bioinformatics Platform) was also used to estimate a phylogeny for the data using the GTR + I + G model, with 500 bootstrap replicates. This resulted in a tree topology (not shown) that did not differ from the Bayesian phylogeny at any nodes with >70% bootstrap support. The tree topology appears to be insensitive to the different models tested.

Systematics

***Anopheles* (*Anopheles*) *barbirostris* van der Wulp**

Anopheles barbirostris van der Wulp, 1884 (♀). Holotype ♀: Mount Ardjoeno, Java, Indonesia (Nationaal Natuurhistorisch Museum, Leiden, The Netherlands).

Anopheles martini Laveran, 1902 (♀). Type locality: near Pursat, Cambodia (Institut Pasteur, Paris).

Anopheles barbirostris innominata Stoker & Waktoedi Koesoemawinangoen, 1949 (♀ ♂). Type locality: Sulawesi, Indonesia (non-extent). Note that this nominal form may be a distinct member of the Barbirostris Complex.

Anopheles (*Anopheles*) *barbirostris* in part (?) of Bonne-Wepster & Swellengrebel, 1953 (Indonesia, ♀* ♂* L*); Reid, 1962 (Indonesia, Malaysia, Thailand, ♀* ♂ L P* E*, taxonomy); Reid, 1968 (Malaysia, Borneo, ♀* ♂* L* P* E, taxonomy, biology); Harrison & Scanlon, 1975 (Thailand, ♀* ♂* L* P*, taxonomy); Reid *et al*., 1979 (Java only, ♀ L P morphology).

Anopheles barbirostris in part (?) of Harrison *et al*., 1988 (Thailand, A L P morphology); Ndoen *et al*., 2010, 2011 (West Timor, Java, ecology, bionomics).

Anopheles barbirostris clade I of Paredes-Esquivel *et al.*, 2009 (COI mtDNA, ITS2 rDNA sequence).

Diagnosis. It is not possible to provide a meaningful morphological diagnosis of *An. barbirostris* at this time because the morphology of other members of the complex, except for the new species described below, has not been studied. Previously published taxonomic articles are limited in the scope of treatment and do not recognize that *An. barbirostris* is a complex of species. However, the diagnostic sequence for the COI region of mtDNA is provided herein for the identification of *An. barbirostris* and one of the previously unnamed species of the complex (see the section on 'DNA sequences').

Female. Large brownish-black mosquito with finely dappled wings and narrowly banded tarsi (Fig. 1).

Head: vertex dark-scaled with patch of pale scales before interocular space; interocular space with mostly dark setae and narrow pale scales. Antenna about two-thirds length of proboscis; pedicel with scales on dorsolateral surface; flagellomere 1 with patch of scales, other flagellomeres without scales. Proboscis length about 2.5 mm, entirely dark-scaled, noticeably shaggy in proximal 0.6–0.7, labella also dark. Maxillary palpus same length as proboscis, entirely dark-scaled, palpomeres 1 and 2 particularly shaggy.

Thorax: scutum with longitudinal bare areas between rather broad lines of golden piliform scales on acrostichal, dorsocentral and marginal areas; anterior promontory with whitish piliform scales medially; posterior segments of acrostichal and dorsocentral areas and entire prescutellar area with covering of golden piliform scales, scutal setae slightly darker and longer than scales; scutellum with golden piliform scales along bases of large golden to golden-brown setae in transverse posterior row. Mesopostnotum and postpronotum bare. Antepronotum with prominent patch of dark scales on dorsoanterior surface and rather sparse fine piliform scales posteriorly. Pleura with golden to golden-brown setae on upper proepisternum, prespiracular area, prealar knob, upper and lower mesokatepisternal areas and upper and middle mesepimeron; scales usually with setae on upper proepisternum, mesokatepisternum and mesepimeron; scales and setae often indistinct on middle of mesepimeron.

Wing: pattern as illustrated, pale scaling as follows – costa often with small humeral pale spot (at least on posterior margin of vein) and usually scattered pale scales between humeral crossvein and small subcostal pale spot, remainder of costa dark to preapical pale spot (= upper apical fringe spot of Reid, 1968) when present (tends to be absent); remigium median pale spot; humeral crossvein with dark scales; vein R with scattered pale scales extending to variable sector pale spot; vein R_1 with apical pale spot adjoining preapical pale spot of costa and usually a line of interspersed pale scales at subcostal area; vein R_2 with subapical pale spot adjoining apical pale spot of R_1; vein R_3 usually with distinct postbasal and preapical pale spots; vein $R_{4+5}$with variable density of interspersed pale scales on median 0.8; vein M_1 with postbasal and preapical pale spots, sometimes with variable pale scaling in between; vein M_2 largely pale-scaled except distally; vein M_{3+4} with mostly pale scaling between basal and apical dark scaling; mediocubital crossvein (mcu) with medial pale spot; vein CuA mainly pale-scaled with distinct short postbasal and apical dark spots, very apex often with few pale scales, postbasal dark spot usually separated by more than its length from medial dark spot on vein lA; vein 1A largely pale-scaled with distinct medial and apical dark spots; narrow pale fringe spots at apices of veins R_{4+5} and CuA.

Halter: integument of scabellum pale; integument of pedicel and capitellum dark and dark-scaled.

Legs: mainly dark-scaled; coxae with small patches of pale scales; forefemur swollen towards base; all femora with few pale scales at base and apex; all tibiae narrowly pale-scaled at apex; foretarsomeres 1 and 2 with narrow apical pale marks or bands, midtarsus entirely dark-scaled, hindtarsomeres 1–4 with narrow apical pale bands or patches that sometimes cross the joints, particularly long tarsomeres 3 and 4.

Abdomen: integument brownish black, sterna paler, especially basally on either side of midline; sterna II–VII with a median patch of pale scales, usually few pale scales forming row along lateral margins and infrequently with scattered pale scales in between lateral rows and median patch, sternum VII with prominent posteromedian tuft of black scales, often few dark scales in same position on sternum VI.

Male. As the female except as follows.

Head: maxillary palpus usually completely dark-scaled, sometimes with indistinct apicolateral pale bands on palpomeres 4 and 5.

Wing: scaling of veins posterior to radius not as dense as in female; small pale fringe spot often at apex of vein R_2.

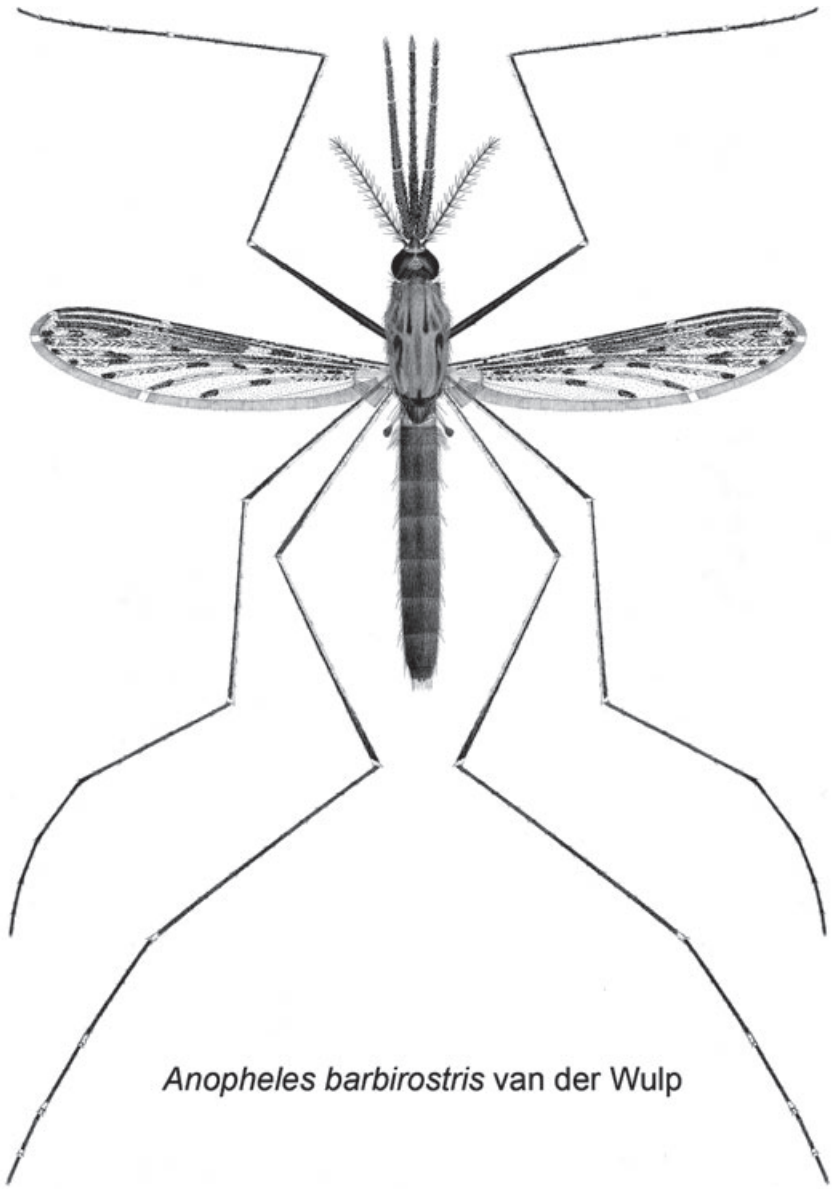

Fig. 1. Female of *Anopheles barbirostris* van der Wulp (dorsal).

Abdomen: tergum VIII with median patch of dark scales, sometimes with few pale scales proximal to dark scales.

Genitalia (Fig. 2C): lateral surface of gonocoxite with dark scales that become longer, more prominent and black toward apex, proximal scaling less distinct with few pale scales sometimes present at base; two parabasal setae borne on small protuberance at base of dorsomesal surface, lateral parabasal about twice as long as mesal one; distinct *inner seta* on middle of ventromesal surface similar to lateral parabasal seta; ventral lobe of claspette small and ill-defined, with two simple setae borne on ventrolateral margin, the more ventromesal one longer than the other; dorsal lobe of claspette with club formed of four or five contiguous and distally fused setae; aedeagus with five or six pairs of leaflets, longest leaflet about half as long as aedeagus, bearing a blunt tooth at base and fine serration mainly on distal two-thirds of both edges, other large leaflets with serration on one or both edges.

Pupa. Lightly to moderately tanned; positions and character of setae as figured, setae usually with ring of darker cuticle at bases (Fig. 2A, B).

Cephalothorax: wings with blurred lattice-like pattern of darker spots; antenna with incomplete dark rings at joints and dark apex; proboscis and fore- and midfemora and -tibiae with blurred rings of darker cuticle.

Trumpet: darkly tanned, with thin uniform rim and deep secondary cleft.

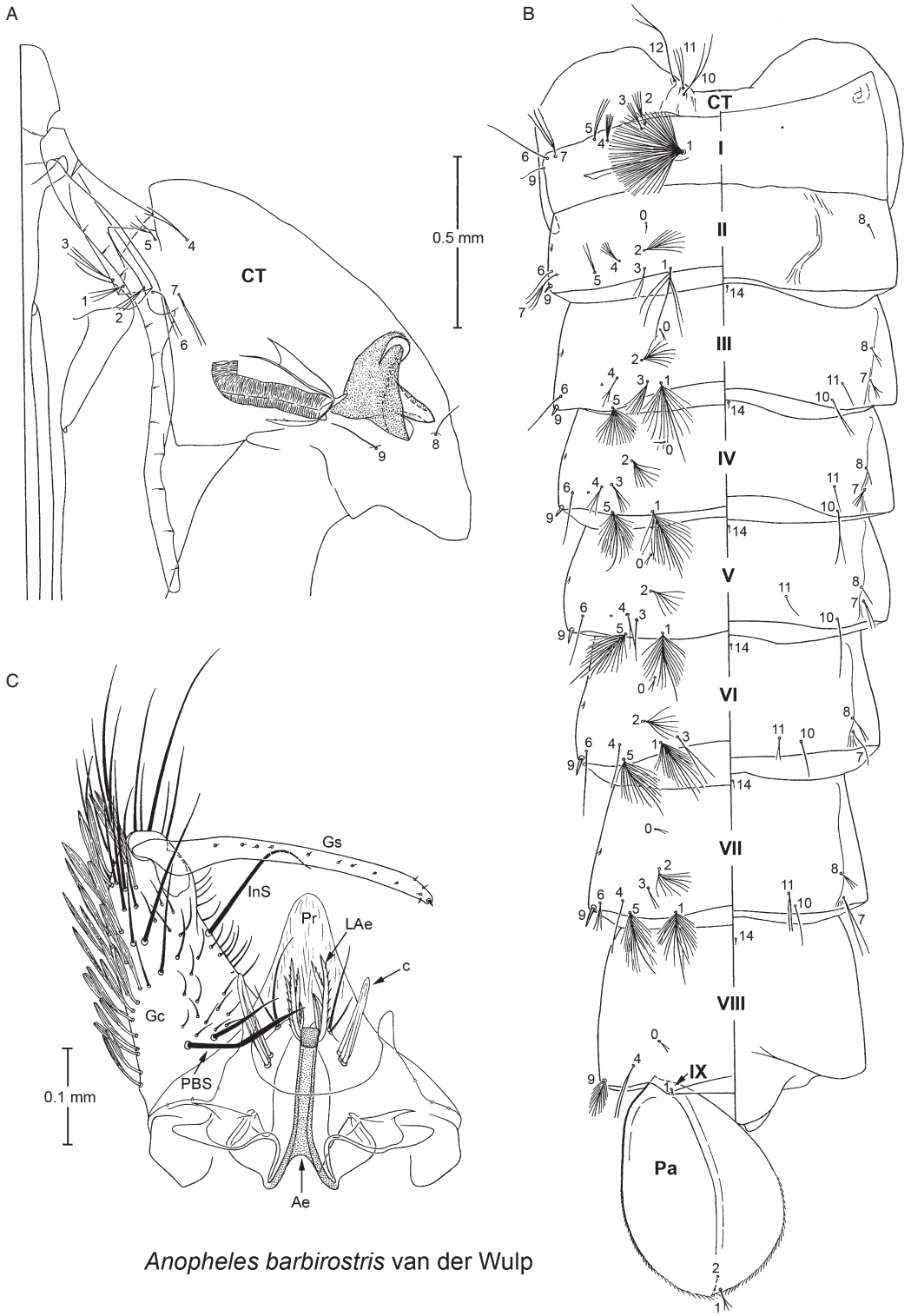

Fig. 2. Pupa and male genitalia of *Anopheles barbirostris* van der Wulp (modified from Harrison & Scanlon, 1975). (A) Pupa, left side of cephalothorax, dorsal to right. (B) Pupa, dorsal (left) and ventral (right) aspects of metathorax and abdomen. (C) Male genitalia, dorsal (tergal) aspect. Ae, aedeagus; c, club on dorsal lobe of claspette; CT, cephalothorax; Gc, gonocoxite; Gs, gonostylus; InS, internal seta; LAe, leaflets of aedeagus; Pa, paddle; PBS, parabasal setae; Pr, proctiger; I–IX, abdominal segments I–IX; 1–14, setal numbers for specified areas, e.g., seta 3-I.

Table 1. Comparison of number of branches of pupal seta 2-III–VII of *Anopheles barbirostris* and *An. vanderwulpi* **sp.n.**

	An. barbirostris		*An. vanderwulpi*	
Seta no.	No. of branches	Sum of pair	No. of branches	Sum of pair
2-III	7–10(8)[a]	14–20(17)	4–7(6)	8–14(12)
2-VI	5–9(7)	10–17(16)	3–6(4)	6–10(8)
2-V	5–9(7)	10–16(15)	4–6(4)	8–10(8)
2-VI	5–7(7)	11–14(14)	3–7(5)	8–12((10)
2-VII	6–9(9)	13–18(15)	4–7(5)	8–12(10)

[a]Range (mode).

Abdomen: lightly to moderately tanned; length about 3.2 mm. Seta 2-II–VII well developed, 2-III–VII with five to 10(7) branches (see Table 1); seta 5-II with one to three (2) branches; setae 1,5-III–VII strongly developed with numerous fine branches (fewer than actual number of branches shown in Fig. 2B), central branch usually distinctly longer than other branches; setae 1,5,9-IV–VII more darkly tanned than integument; seta 8-II present or absent.

Paddle: with darkly tanned base and spot of darker cuticle around insertions of setae 1,2-Pa, refractile margin approximately 0.75 length of paddle; seta 1-Pa usually single with apex split into two to four elements.

Larva, fourth-instar. Generally darkly pigmented; positions and character of setae as illustrated (Fig. 3).

Head: about as wide as long; unevenly tanned, with mottled pattern of darker cuticle, collar and dorsomentum darkly tanned. Seta 2-C simple; seta 3-C broom-like, with 41–53 stiff branches arising from distinct basal stem, sum of branches of both 3-C = 80–100; seta 4-C small, with two to four branches; setae 6,7-C often with 16–19 branches; setae 8,9-C usually with seven to ten branches.

Antenna: moderately tanned; mesal and ventral surfaces strongly spiculate; length about 0.4 length of head.

Thorax: integument hyaline, smooth. Seta 1-P often with five to eight branches from near base; seta 11-P with two to four simple branches arising from short basal stem; seta 14-M commonly with 12–16 branches; seta 3-T palmate with pale lanceolate leaflets; seta 8-T normally with 26 or 27 branches.

Abdomen: integument smooth except for fine spicules on mid-ventral areas of segments II–VIII. Seta 1-I similar to seta 3-T; seta 1-II–VII fully palmate, leaflets with blades darkly tanned proximal to step-like margins of tapered terminus; seta 13-I often with 12 or 13 branches; seta 2-II generally with four or five branches; seta 5-III frequently with six to eight branches; seta 13-IV usually with three or four branches; seta 9-VII often with three or four branches. Pecten plate darkly tanned, usually with 10 or 11 long spines; membrane posterior to saddle with numerous relatively long spicules; seta 1-X about as long saddle.

DNA sequences. Specimens identified as *An. barbirostris* (clade I of Paredes-Esquivel *et al.*, 2009) are shown in Table S1, together with GenBank Accession numbers for the mtDNA COI sequences. The results of Bayesian analysis of COI sequences from specimens of *An. barbirostris* and other members of the Barbirostris Complex are shown in Fig. 4. The ITS2 subunit for clade I yields a dominant product of 1545 bp. For all specimens where both COI sequences and ITS2 products were obtained, the results were wholly congruent. Specimens of this clade were found in both Central Java and East Java, including villages close to the type locality.

Taxonomy. We examined the sole type specimen (holotype female) of *An. barbirostris* in the Nationaal Natuurhistorisch Museum in Leiden, The Netherlands, but it was not possible to distinguish it from females of either clade I or clade II of Paredes-Esquivel *et al*. (2009), which were collected in the vicinity of the type locality of Mount Ardjoeno (*Gunung Arjuno*) located in East Java Province, Indonesia. The specimen is very badly damaged, with missing appendages and the abdomen detached and glued to the platform supporting the specimen. It was collected more than 128 years ago.

From our limited sampling (due to the collection of progeny broods), clade I is more common in eastern Java than clade II. Three-quarters of specimens collected by us in the vicinity of the type locality of *An. barbirostris* in East Java Province (Singosari and Karangploso Subdistricts) belong to clade I (sympatric with clade II in one village). In comparison, three-quarters of specimens collected in Central Java Province (Krajan Hamlet in Jambu District) were from clade II. The species represented by clade I is much more common, being found not only in Java but also in South Kalimantan and extensively in Thailand (Paredes-Esquivel *et al*., 2009). Records of clade II are fewer but it has been recorded from Padang Cermin, Lampung Selatan Province, southern Sumatra, Indonesia (incorrectly listed as 'Padang Cermin-Lampung, West Sumatra' in Paredes-Esquivel *et al*., 2009).

It is not possible to determine which clade was collected and named *An. barbirostris* by van der Wulp (1884). Because the holotype of *An. barbirostris* is very old (unlikely to yield useful DNA), in very poor condition (not amenable to destructive sampling) and cannot be identified as either clade I or clade II, we feel that in order to promote nomenclatural stability, it is justifiable to restrict the inveterate concept of *An. barbirostris* s.s. to the more common and widely distributed clade I.

Bionomics. Based on specimens identified as clade I (Table S1), *An. barbirostris* occurs in hilly country, often in the proximity of rice fields. Adult females appear to be mainly zoophilic. Adults have frequently been collected resting in shelters for cows and horses, and in vegetation near these shelters. The immature stages are found among emergent and floating vegetation in clear, stagnant or slowly running bodies of fresh water associated with rice cultivation. Individual aquatic habitats may be in full sunlight or partially shaded (Takken *et al.*, 1991).

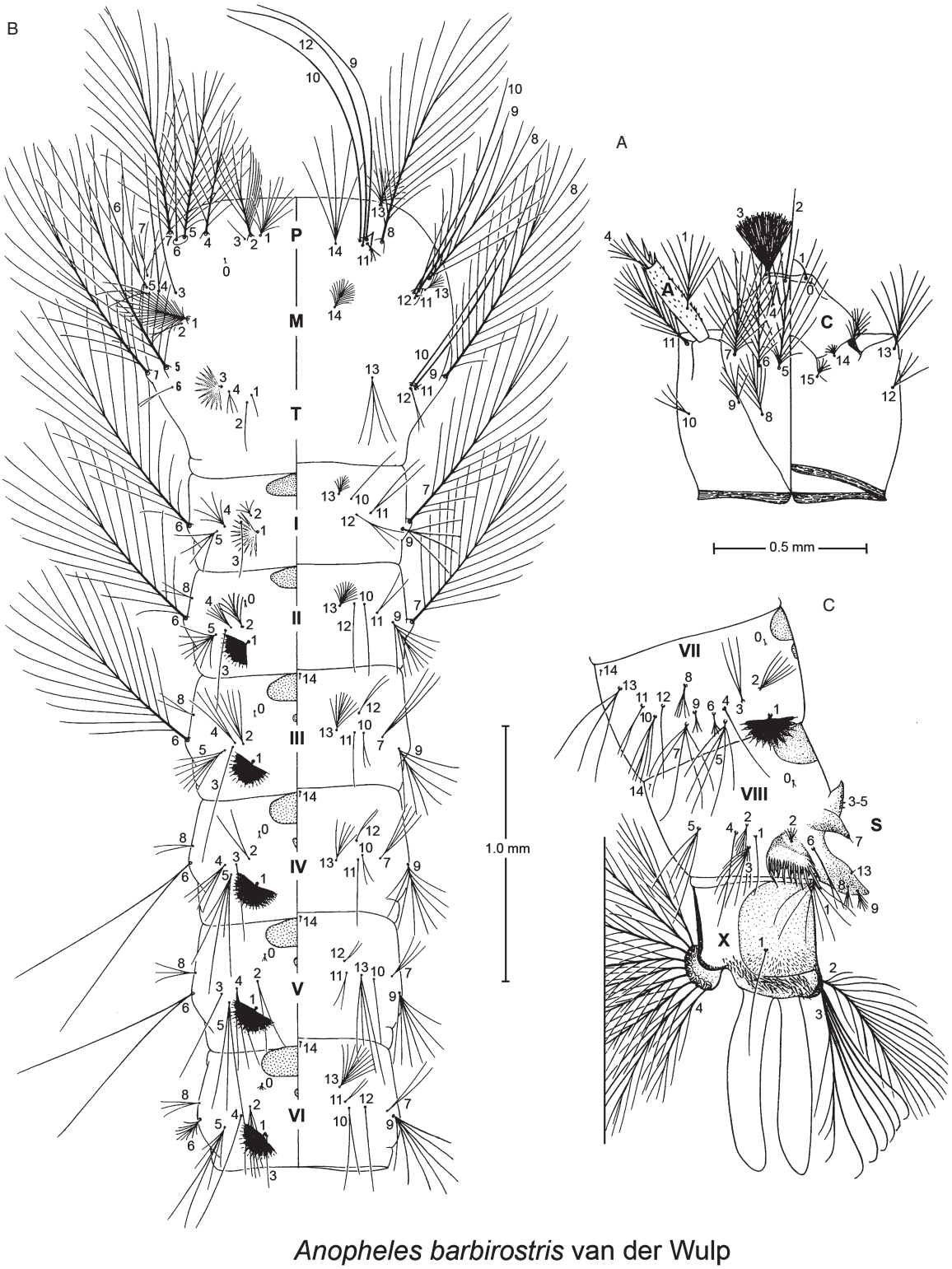

Fig. 3. Fourth-instar larva of *Anopheles barbirostris* van der Wulp (modified from Harrison & Scanlon, 1975). (A) Head, dorsal (left) and ventral (right) aspects of left side. (B) Thorax and abdominal segments I–VI, dorsal (left) and ventral (right) aspects of left side. (C) Abdominal segments VII–X, left side. A, antenna; C, cranium; P, prothorax; M, mesothorax; S, spiracular lobe; T, metathorax; I–VIII, X, abdominal segments I–VIII and X; 1–15, setal numbers for specified areas, e.g., seta 5-C.

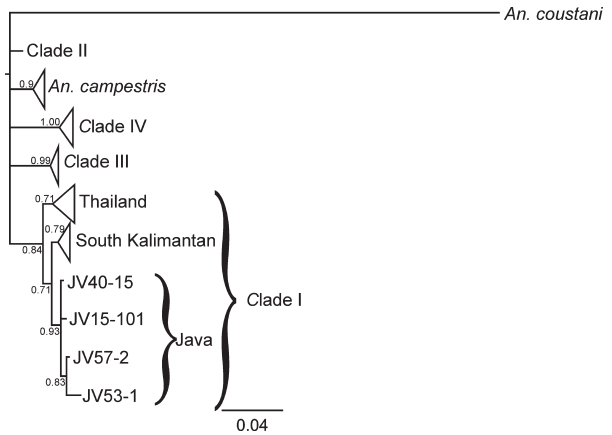

Fig. 4. Bayesian consensus tree for cytochrome oxidase I sequences. *Anopheles coustani* has been used to root the tree. Clade I, *Anopheles barbirostris* van der Wulp; clade II, *Anopheles vanderwulpi* **sp.n.**; clades III and IV, clades of *An. barbirostris s.l.* described in Paredes-Esquivel *et al.* (2009). Clades I, III and IV from Thailand and South Kalimantan have been collapsed to triangles of fixed height for clarity. The numbers at the nodes are the Bayesian posterior probabilities.

Distribution. Based on the COI sequence and the characteristic size of ITS2: Indonesia (East and Central Java provinces; present study), Indonesia (West Java and South Kalimantan provinces), Thailand (Mae Hong Song Province) (Paredes-Esquivel *et al*., 2009).

Material examined. Specimens unequivocally identified as *An. barbirostris* from COI sequence (clade I of Paredes-Esquivel *et al*., 2009) are listed in Table S1. Specimens examined that could not be or were not identified as either *An. barbirostris* or the new species described below are listed in Table S2.

Anopheles (*Anopheles*) *vanderwulpi* Townson & Harbach, sp.n.

Anopheles (*Anopheles*) *barbirostris* in part (?) of Bonne-Wepster & Swellengrebel, 1953 (Indonesia, ♂* ♀* L*); Reid, 1962 (Indonesia, ♂ ♀* L P* E*, taxonomy); Reid *et al*., 1979 (Java only, ♀ L P morphology).

Anopheles barbirostris in part (?) of Ndoen *et al*., 2010, 2011 (West Timor, Java, ecology, bionomics).

Anopheles barbirostris clade II of Paredes-Esquivel *et al*., 2009 (COI mtDNA ITS2 rDNA sequence).

Diagnosis. Sequences for the COI gene of mtDNA and the characteristic length of ITS2 in clade II are diagnostic and reliably distinguish *An. vanderwulpi* from other members of the Barbirostris Complex (Paredes-Esquivel *et al*., 2009). For practical purposes, *An. vanderwulpi* is morphologically indistinguishable from *An. barbirostris*. No characters were found during comparative morphological studies of COI-typed progeny broods of *An. vanderwulpi* and *An. barbirostris* obtained from females collected around the type locality of the latter species that would consistently and reliably distinguish the species. Some minor morphological features are denoted below, but a more detailed comparative study of the adult, larval and pupal stages of all species of the Barbirostris Complex from different geographical populations is needed to establish whether these or other characters may be useful for distinguishing the species. For the time being, ecological and behavioural studies of the species must rely on molecular methods of identification.

Female. As described for *An. barbirostris*, inseparable morphologically. Preapical pale spot of costa always (?) present.

Male. Not studied in detail due to paucity of specimens.

Pupa. As described for *An. barbirostris*. Differences in the number of branches of seta 2-II–VII are shown in Table 1.

Larva. As described for *An. barbirostris*. Seta 3-C with 19–38 branches, sum of branches of both 3-C = 43–74; seta 8-T normally with 19-21 branches.

DNA sequence. Specimens identified as *An. vanderwulpi* (clade II of Paredes-Esquivel *et al.*, 2009) are shown in Table S3, together with GenBank accession numbers for mtDNA COI sequences. The results of Bayesian analysis of COI sequences from specimens of *An. vanderwulpi* and other members of the Barbirostris Complex are shown in Fig. 4. The ITS2 subunit for clade II yields a dominant product of 1727 bp. Again, for specimens where both COI sequences and ITS2 products were obtained, the results were wholly congruent. Specimens of clade II were found in both Central Java and East Java provinces, including villages close to the type locality of *An. barbirostris.*

Bionomics. Records for COI-typed specimens of *An. vanderwulpi* collected in East and Central Java provinces indicate that this species occurs in the same hilly environments as *An. barbirostris*. However, clade II has also been identified from locations very near the coastline in Pelabuhan Ratu, West Java and Padang Cermin, southern Sumatra (Paredes-Esquivel *et al.*, 2009). Adult females also appear to be mainly zoophilic. Adults have been found resting in animal shelters (for water buffalo) and surrounding vegetation. Larvae and pupae occur in the same types of habitats as those of *An. barbirostris*, but collections so far do not indicate whether they may occupy the same or different niches.

Distribution. Indonesia: Java, East Java Province, Central Java Province (present study), West Java Province, and Lampung Selatan Province, southern Sumatra (Padang Cermin) (Paredes-Esquivel *et al*., 2009) (see also 'Discussion').

Etymology. Anopheles vanderwulpi is named in honour of Frederik Maurits van der Wulp (1818–1899), who, in addition to describing the nominotypical member of the Barbirostris Complex, described nearly 2000 species of Diptera from all over the world.

Type series. The type series of *An. vanderwulpi* consists of the specimens listed in Table S3. Holotype ♀ (JV67-5) – sibling of wild-caught female with following collection data: INDONESIA, Central Java Province, Semarang District, Jambu Subdistrict, Klurahan, Krajan Hamlet, S 07°17′03″E 112°21′53″, animal shelter, 14 November 2007 (McAlister *et al*.). The series is deposited in the NHM, London. Specimens examined that could not be or were not identified as either *An. barbirostris* or the new species are listed in Table S2.

Discussion

A total of 465 formally named species are currently recognized as distinct morphological and/or genetically defined species of the genus *Anopheles* Meigen. These species are divided between seven subgenera: *Anopheles* (cosmopolitan, 182 species), *Baimaia* Harbach, Rattanarithikul & Harrison (Oriental, one species), *Cellia* Theobald (Old World, 220 species), *Kerteszia* Theobald (Neotropical, 12 species), *Lophopodomyia* Antunes (Neotropical, six species), *Nyssorhynchus* Blanchard (Neotropical, 39 species) and *Stethomyia* Theobald (Neotropical, five species) (Harbach, 2012). Four of the subgenera, *Anopheles*, *Cellia*, *Kerteszia* and *Nyssorhynchus*, include the species that transmit human malarial parasites. Most *Anopheles* vector species have been found to comprise complexes of sibling species. Our future understanding of the genetics of *Anopheles* species complexes is likely to be improved by the development of mosquito genome databases such as those included in the bioinformatics database VectorBase (www.vectorbase.org), which currently includes 16 *Anopheles* species (see also Megy *et al.*, 2012).

The classification of genus *Anopheles* Meigen, including species complexes, was reviewed by Harbach (1994, 2004), and an up-to-date version is available online (Harbach, 2012). Comprehensive information on the dominant malaria vectors of the world, most of which are members of sibling species complexes, is summarized in Sinka *et al*. (2012), and information pertaining to the Barbirostris Complex and other malaria vector taxa in Asia is reviewed in Sinka *et al*. (2011). At present, 94 formally named species of *Anopheles* are members of sibling species complexes. Eighteen of these species taken together actually comprise a total of 53 species. Excluding nominotypical species, 44 of these have yet to be recognized with formal Latin names. With few exceptions (Mattingly, 1977; Harbach *et al*., 2007), no formal names have been given to members of sibling species that may be conspecific with nominal species that are currently regarded as synonyms of the nominotypical species. In those cases where sibling species have been given formal Latin names, e.g. the Australasian Farauti Complex of subgenus *Cellia* (Schmidt *et al.*, 2001, 2003), there were no junior synonyms to consider when naming the species. In cases involving junior synonyms, e.g. the Culicifacies Complex of subgenus *Cellia* (Kar *et al*., 1999), it will be necessary to obtain DNA sequence from type specimens or topotypic material to determine the availability of the synonymous names for members of sibling species complexes.

The present study has focused on the type locality of *An. barbirostris* in Java, but the studies of Satoto (2001) suggest that further studies in Flores may yield both *An. barbirostris* and *An. vanderwulpi*, and an additional species may occur in Sulawesi. Unfortunately, the COI sequences acquired by Satoto were from a different part of the mtDNA COI sequence than that chosen by Paredes-Esquivel *et al.* (2009), and this makes comparisons difficult. The COI sequence in insects has been divided into 25 regions comprising five structural classes that evolve at different rates (Lunt *et al*., 1996). The region amplified in the present study (based on that of Paredes-Esquivel *et al.*, 2009) comprises a fragment extending from internal loop 3 to the COOH terminal region, a fragment that includes, according to Lunt *et al*. (1996), some of the most variable regions.

Satoto (2001) sequenced the COI of three specimens from each of six localities: two in Sulawesi, two in Flores and two in Java. It seems possible that one of his putative species, X or W, corresponds to *An. vanderwulpi* and the other to *An. barbirostris*. There is a need for further studies in Sulawesi and throughout the Lesser Sunda Islands (in particular, Sumbawa, Sumba, Flores, Alor, West Timor and Timor-Leste) to establish the number of species within the Barbirostris Subgroup in Indonesia. We anticipate that the results of this study will facilitate such further study.

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/j.1365-3113.2012.00653.x

Table S1. Indonesian specimens identified as *Anopheles barbirostris* s.s. (clade I of Paredes-Esquivel *et al*., 2009) based on COI sequence and examined during the study.

Table S2. Indonesian specimens examined and available for further study that could not be or were not identified as either *Anopheles barbirostris* or *An. vanderwulpi*.

Table S3. Indonesian specimens identified as *Anopheles vanderwulpi* **sp.n.** (clad II of Paredes-Esquivel *et al*., 2009) based on COI sequence and examined during the study. These specimens comprise the type series of the species (holotype JV67-5; others paratypes).

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