



## ***Bothaella manhi*, a new species of tribe Aedini (Diptera: Culicidae) from the Cuc Phuong National Park of Vietnam based on morphology and DNA sequence**

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### **Abstract**

A new species of genus *Bothaella* (Diptera: Culicidae) collected along with two other species of the genus during surveys for flavivirus isolations in the Cuc Phuong National Park in northern Vietnam is formally described and named as *Bothaella manhi*, **sp. n.** The adults, pupa and fourth-instar larva are characterized, the male genitalia and the two immature stages are illustrated and DNA sequence data are included for regions coding for sections of the COI and COII genes (mtDNA). The species is compared and distinguished from the other species of the genus, and sequence data are used to hypothesise its phylogenetic relationship with *Bo. helenae* and *Bo. kleini*, the other two species collected during the survey.

**Key words:** *Bothaella alongi*, *Bothaella helenae*, *Bothaella kleini*, COI, COII, mosquito, new species, taxonomy

### **Introduction**

*Bothaella* Reinert, 1973 is a little-known genus of mosquitoes (Diptera: Culicidae) that includes five species in Southeast Asia: *Bo. alongi* (Galliard & Ngu, 1947), *Bo. brownscutuma* (Dong, Zhou & Dong, 1999), *Bo. eldridgei* (Reinert, 1973), *Bo. helenae* (Reinert, 1973) and *Bo. kleini* (Reinert, 1973). Two of these species, *Bo. helenae* and *Bo. kleini*, and an unidentifiable species were found among adult mosquitoes collected in the Cuc Phuong National Park of Ninh Binh Province in northern Vietnam for flavivirus screening in August 2008. Additional specimens of *Bo. helenae* and the unidentifiable species were subsequently found in a collection of individually reared mosquitoes made in the park in July 2000. Dissection of the male genitalia confirmed that the unidentifiable species is distinct from *Bo. brownscutuma*, *Bo. eldridgei*, *Bo. helenae* and *Bo. kleini*, and a critical comparison of specimens with the detailed descriptions of the female and larva of *Bo. alongi* (Reinert *et al.*, 2006), the male of which is unknown, revealed it is distinct from that species as well. Based on these findings, the species is formally described and named as new in this paper. Sequence data for regions coding for sections of the COI and COII genes of mitochondrial DNA were obtained from specimens of the new species, *Bo. helenae* and *Bo. kleini* and used to hypothesise their phylogenetic relationships.

### **Material and methods**

This study is based on wild-caught adults (vouchers of mosquitoes used for flavivirus screening) and adults reared from larvae and/or pupae collected in the Cuc Phuong National Park, Ninh Binh Province, northern Vietnam.

**Morphology.** Observations of adult mosquitoes were made under simulated natural light. Larval and pupal chaetotaxy were studied using differential interference contrast microscopy. Numbers in parentheses represent modes, when apparent, of the recorded ranges. The morphological terminology and abbreviations

used in the descriptions and illustrations, respectively, are derived from the Anatomical Glossary of the online Mosquito Taxonomic Inventory (<http://mosquito-taxonomic-inventory.info/anatomical-glossary-overview>). The symbols ♀, ♂, Le and Pe used in the **Type series** represent female, male, larval exuviae and pupal exuviae, respectively.

**DNA sequences.** Sequences of regions coding for sections of the COI and COII genes (mtDNA) were generated from adults and adults reared from larvae collected in the Cuc Phuong National Park. In addition to the three *Bothaella* species of particular interest from this specific locality, sequences were obtained from specimens representing a range of Culicidae collected in Thailand and Vietnam. One or more legs were taken from individual pinned mosquitoes that had been stored at  $-80^{\circ}\text{C}$  or dried over silica gel and homogenised as per Cook *et al.* (2006). A 60- $\mu\text{l}$  aliquot of the homogenate was added to 125  $\mu\text{l}$  of Chelex 100 Resin 15% w/v (BioRad, Hercules, USA), incubated at  $95^{\circ}\text{C}$  for 20 min and centrifuged for 10 min at maximum speed. A 60  $\mu\text{l}$  aliquot of supernatant was buffered using 10X PCR buffer (Roche Diagnostics, West Sussex, UK), and of this extraction 5  $\mu\text{l}$  was used in 25- $\mu\text{l}$  PCR reactions. All remaining homogenates and extractions were stored at  $-80^{\circ}\text{C}$  and  $-20^{\circ}\text{C}$ , respectively.

The COI gene was amplified using primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al.*, 1994) with a primer concentration of 10  $\mu\text{M}$  and reaction conditions of 5 min at  $95^{\circ}\text{C}$ , 40 cycles of 30 sec at  $95^{\circ}\text{C}$ , 30 sec at  $48^{\circ}\text{C}$ , 45 sec at  $72^{\circ}\text{C}$ , followed by final extension time of 5 min at  $72^{\circ}\text{C}$ . Identical reaction conditions were used to amplify the COII gene using the modified primers SCTL2-J-3037 (5'-ATGGCAGATTAGTGCAATGA-3') and TK-N-3785 (5'-GTTTAAGAGACCAGTACTTG-3') (Liu & Beckenbach, 1992).

Only specimens from which clean forward and reverse sequences for both the COI and COII regions were obtained were included in phylogenetic analyses, with the exception of two specimens of *Bo. kleini* for which only COI sequence was obtained. Sequencher v. 4.8 (Gene Codes Corporation, Ann Arbor, USA) was used to combine reverse and forward sequences from each mosquito with primers trimmed. Final datasets were compiled using Se-Al (available at <http://evolve.zoo.ox.ac.uk/software/Se-Al/main.html>). Nucleotides were aligned using MUSCLE (Edgar, 2004). Maximum likelihood (ML) phylogenetic trees were estimated via Bayesian methods in MrBayes v. 3.1.2 (Huelsenbeck & Ronquist, 2001) with a minimum of 50 million generations, a burnin of 10% and assessment of stationarity at effective sample size (ESS) > 400 using Tracer v. 1.4.1 (part of the BEAST package, Drummond & Rambaut, 2007). Sequences were submitted to GenBank (accession numbers are listed below).

## Taxonomy

### *Bothaella manhi* Harbach & Cook, new species

**Diagnosis.** The adults of *Bo. manhi* have pale bands on the hindtarsi (distinction from *Bo. kleini*) and a patch of silvery scales on the postgena (distinction from *Bo. alongi*). The male of *Bo. alongi* is unknown, but the presence of a sigmoidal seta on the basal mesal lobe of the male genitalia distinguishes *Bo. manhi* from the other species of the genus. The pupa of *Bo. manhi* resembles the pupa of *Bo. helenae* and differs from the pupae of *Bo. eldridgei* and *Bo. brownscutuma* (pupae of *Bo. alongi* and *Bo. kleini* are unknown) in having a multiple-branched, stellate-like seta 1-Pa. The fourth-instar larva of *Bo. manhi* is distinguished from other members of the genus (the larva of *Bo. kleini* is unknown) by one or more of the following characters: seta 4-C with 6 or fewer branches (distinction from *Bo. alongi* and *Bo. eldridgei*), setae 1-M,T and 1-VIII with 1-4 branches and seta 5-VIII single or double (distinctions from *Bo. alongi* and *Bo. helenae*) and siphon index greater than 4.0 (distinction from *Bo. alongi*, *Bo. eldridgei* and *Bo. brownscutuma*).

Sequences for the COI and COII regions of mtDNA distinguish *Bo. manhi* from *Bo. helenae* and *Bo. kleini*. Sequence data are unavailable for the other members of the genus, but the phylogenetic relationships inferred by the COI and COII sequences suggest these regions are likely to be diagnostic for each species.

**Female.** Overtly dark brown to black with areas of silvery-white scales. *Head:* Vertex with broad dark decumbent scales posteriorly and large triangular patch of broad silvery scales anteriorly that extend onto

interocular space; occiput with transverse row of dark erect scales; postgena with small patch of silvery scales; ocular line narrow with broad dark scales, few dark ocular setae; 2 dark interocular setae. Compound eyes continuous above antennal pedicels. Clypeus bare. Antenna slightly longer than proboscis, pedicel with large mesal patch of broad silvery scales, flagellar whorls with relatively few setae, flagellomere 1 with dark scales mesally. Maxillary palpus length about 0.4 mm, slightly less than 0.2 length of proboscis, dark-scaled. Proboscis narrow, length about 2.3 mm, slightly longer than forefemur, dark-scaled. *Thorax*: Integument dark brown, scutum covered with narrow dark falcate scales except prescutellar area and supraalar area adjacent to posterior dorsocentral and prescutellar setae bare; dark setae on middle and lateral areas of anterior promontory, scutal fossa, prescutellar and antealar areas, anterior and posterior dorsocentral areas, supraalar area, lateral prescutellar line and 1 on parascutellar area; paratergite narrow, bare. Scutellum with broad silvery scales on midlobe, few broad dark scales on lateral lobes, several dark setae on all lobes. Mesopostnotum bare. Anteprepronotum with patch of broad silvery-white scales dorsally and line of dark setae anterior and lateral to scale patch. Postpronotum without scales, 2 or 3 dark setae posteriorly. Pleura with few dark setae: 2 upper proepisternal, few prealar, 2,3(3) mesokatepisternal, few upper and 1 lower mesepimeral; with prominent patches of broad silvery scales on upper proepisternal, postspiracular, upper mesokatepisternal, lower mesokatepisternal and upper mesepimeral areas. *Wing*: Length about 0.6 mm; entirely dark-scaled; alula with row of narrow to slightly broad scales on margin; upper calypter with row of setae on upper margin; vein R2+3 slightly shorter than vein R2. *Legs*: Forecoxa with patches of broad silvery scales anteriorly at base and apex and dark scales adjacent to basal patch; mid- and hindcoxa with patch of silvery scales on anterior side of lateral midline; ventral surface of foretrochanter dark-scaled with silvery scales at apex, ventral surface of mid- and hindtrochanter with silvery scales; forefemur entirely dark-scaled except for inconspicuous silvery scales at apex of anterior surface, midfemur dark-scaled with small dorsoanterior patch of silvery scales at apex, proximal 0.5–0.6 of hindfemur silvery-scaled, this area sometimes with few thinly scattered dark scales on dorsal midline, apex with dorsoanterior patch of silvery scales, dark scaling sharply contrasted between proximal and apical silvery areas; tibiae and foretarsus dark-scaled, midtarsus with dorsal spots of white scales at bases of tarsomeres 1–3, hindtarsus with basal and apical bands of white scales on tarsomeres 1–4, tarsomere 5 white-scaled; fore- and midungues equal, each with 1 tooth, hindungues equal, both simple. *Abdomen*: Dark-scaled, laterotergite of tergum I with patch of silvery scales, terga II–VII with basolateral patches of silvery scales; sterna dark-scaled with basal bands of silvery scales confluent with basolateral patches of terga. *Genitalia*: Proximal 0.7 of tergum and sternum VIII retracted into segment VII, both wider than long; tergum VIII length 0.18 mm, width 0.36 mm, index 0.49, posterior margin slightly concave, with setae and scales on caudal 0.4, basolateral seta minute; sternum VIII length 0.28 mm, width 0.38 mm, index 0.75, posterior margin produced on either side of midline, scales and setae on caudal 0.8, setae denser and scales absent on posteromedian area, setae 1–5-S in more or less diagonal line. Tergum IX length 0.09 mm, width 0.16 mm, index 0.45, posterior margin with shallow median emargination, 2–4 setae distally on either side of midline, 4–8 total setae; insula tongue-like, long, with 1–3 minute tuberculi distally. Postgenital lobe moderately long, apex with deep median emargination (0.36 of dorsal length), dorsal surface with 2 distal setae on either side of emargination, ventral surface with 5–7 setae on either side of midline, 14–18 total setae, dorsal length 0.06–0.07 mm, dorsal width 0.07 mm, ventral length 0.12 mm, ventral width 0.06 mm, dorsal postgenital lobe index 0.86–1.08, ventral postgenital lobe index 2.0, ventral postgenital lobe/cercus index 0.75. Cercus moderately long, apex rounded, inner margin more or less straight, with setae on distal 0.4 of dorsal and lateral surfaces, scales absent, dorsal length 0.16 mm, width 0.07 mm, cercus index 2.29, cercus/dorsal postgenital lobe index 2.29–2.83. Three spherical spermathecal capsules, 1 slightly larger than the other two, few small spermathecal capsule pores near orifices.

**Male.** Similar to female except for obvious sexual differences; other differences include the following. *Head*: Proboscis slightly shorter, about 2.1 mm, about 1.05 length of forefemur. *Wing*: Scaling reduced on veins posterior to R-R1. *Genitalia* (Fig. 1C): Tergum IX relatively broad, 2–5 setae on either side of midline (5–10 total setae), most lateral seta on either side inserted distinctly anterior to the others, which are usually in a more or less straight transverse line at posterior margin. Sternum IX large, with 3–7 (mode 5) setae inserted posteromedially. Gonocoxite relatively short and broad, mesal surface membranous, ventral, lateral and

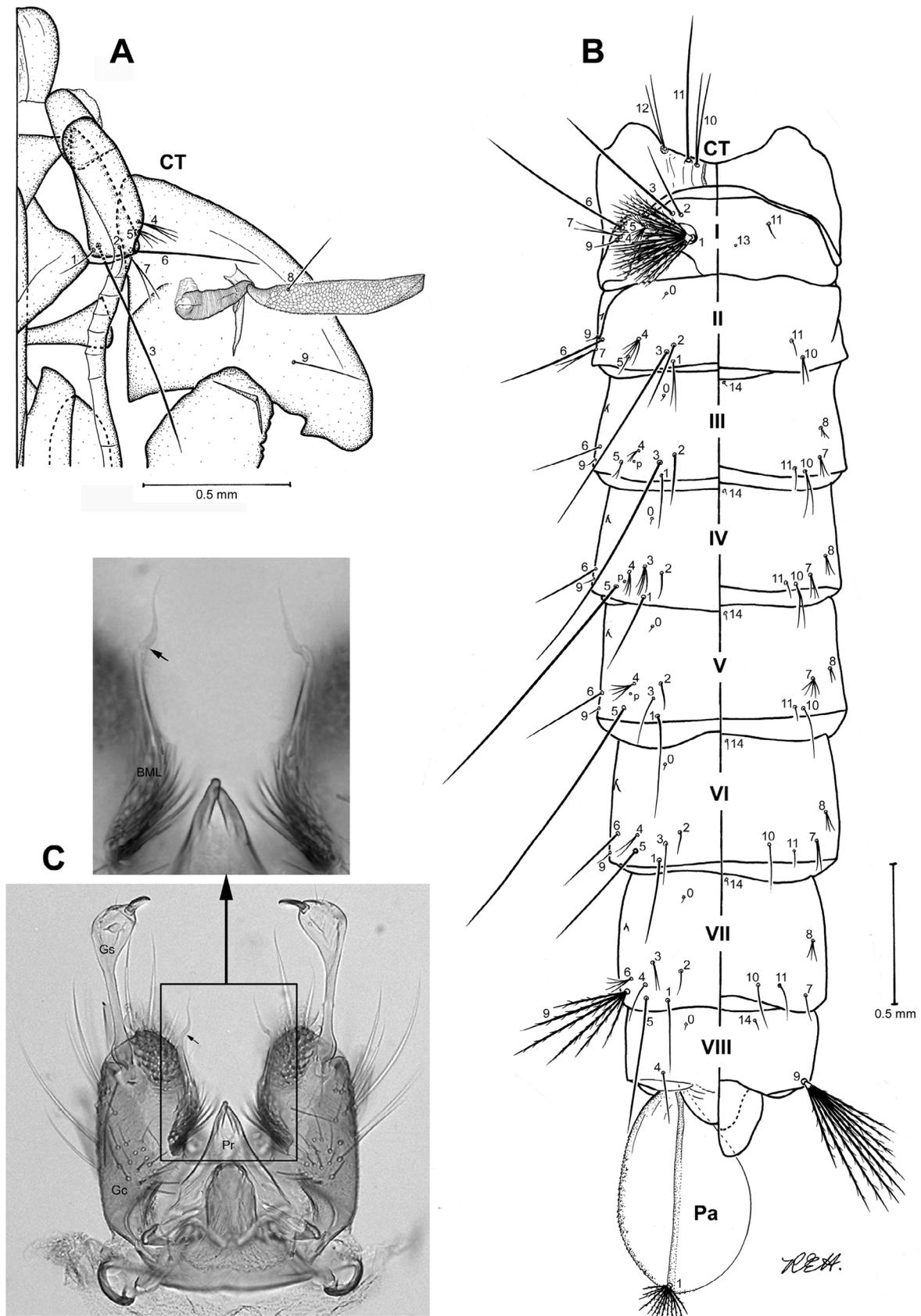
dorsomesal surfaces with numerous setae, dorsomesal setae generally thinner more flexible and in distinct patch, dorsolateral setae interspersed with scales, upper ventromesal area with dense patch of foliform setae (resembling scales but without longitudinal ridges). Gonostylus long, about 0.8 length of gonocoxite, apical 0.45 expanded with rounded apex, expanded portion with 3–5 dorsal setae and a single subapical ventrolateral seta; gonostylar claw tongue-like, borne apically. Basal mesal lobe expanded and elongate apically, with numerous tapered and lanceolate setae, caudal end of lobe slightly produced and bearing a lanceolate seta and a much longer flattened sigmoidal seta. Proctiger gradually tapered distally to blunt apex, cercus membranous, cercal setae absent; tergum X divided into 2 narrow sclerites borne laterally on either side at base of proctiger. Aedeagus comprised of 2 lateral, apically joined sclerites, ventral margins of sclerites thickened, ridge-like and weakly denticulate distally.

**Pupa.** Character and positions of setae as illustrated in Fig. 1A,B; numbers of branches in Table 1. *Cephalothorax*: Lightly tanned, scutum and metanotum darker; setae 3,6-CT very long and normally single. *Trumpet*: Angusticorn, more or less tubular, slightly bent anteriorly at tracheoid, moderately tanned, pinna without slit; length 0.52–0.73 mm (mean 0.61 mm); pinna fairly long, 0.11–0.24 mm (mean 0.17 mm); width at midlength 0.07–0.15 mm (mean 0.11 mm); index 4.40–8.57 (mean 5.89). *Abdomen*: Length 2.70–4.21 mm (mean 3.35 mm); lightly to moderately tanned, tergum I and anterior margins of terga and sternae II–VIII darker. Setae 3-I–III, 6-I and 5-IV,V very long, single (1 of 20 seta 6-I double), about twice length of following tergum; seta 2-II–VII normally inserted distinctly anteromesad of seta 1; seta 6-VII inserted more or less directly anterior to seta 9; seta 9-VII,VIII strongly developed, longer than segment VIII, with stiff aciculate branches; seta 10-II absent; seta 11-I usually single, occasionally double, often absent; seta 13-I usually absent or represented by an empty alveolus, minute and single if present. *Genital lobe*: Length about 0.2 mm in female; about 0.3 mm in male. *Paddle*: Longer than broad, ovoid, length 0.66–0.83 mm (mean 0.76 mm), width 0.40–0.51 mm (mean 0.45 mm), index 1.60–1.82 (mean 1.70); midrib long, distinct to seta 1-Pa; outer margin minutely spiculate on distal 0.75, inner margin minutely spiculate on distal 0.25. Seta 1-Pa relatively long, more or less stellate, with 9–13(10) minutely aciculate branches; seta 2-Pa absent.

**TABLE 1.** Range (mode) of branches for pupal setae of *Bothaella manhi* obtained from 10 pupal exuviae (20 setae).

Setae no.	Cephalo-thorax		Abdominal segments									Paddle	
	CT		I	II	III	IV	V	VI	VII	VIII	IX	Pa	
0	–	–	1	1	1	1	1	1	1	1	–	–	
1	1,2(1)	7–14(11) <sup>a</sup>	1–4(2)	1,2(1)	1,2(1)	1,2(1)	1,2(1)	1,2(1)	1,2(1)	–	–	9–14(10)	
2	1,2(1)	1–3(1)	1,2(1)	1,2(1)	1–3(1)	1,2(1)	1–3(1)	1	–	–	–	–	
3	1,2(1)	1	1	1	2–5(4)	1,2(1)	1,2(1)	1–3(2)	–	–	–	–	
4	2–5(3)	4–7(5,6)	4–7(6)	2–4(3)	2–4(2)	3–6(4)	2,3(2)	1,2(1)	1,2(1)	–	–	–	
5	2–5(3)	1–3(2)	1,2(1)	1–3(2)	1	1	1,2(1)	1,2(1)	–	–	–	–	
6	1	1,2(1)	1,2(1)	1	1,2(1)	1	1–3(1)	3–7(4)	–	–	–	–	
7	1–4(2)	1–4(2)	2–4(3)	2–7(3)	2–5(3)	4–6(5)	1–3(2)	1,2(1)	–	–	–	–	
8	1–4(1)	–	–	2–4(3)	2–4(3)	2–4(2)	2–5(3)	2–5(3)	–	–	–	–	
9	1–3(1)	1,2(1)	1	1	1	1	1	4–9(8)	8–13(9)	–	–	–	
10	1–3(1)	–	0–2 <sup>d</sup>	2,3(2)	1–5(2)	1,2(1)	1,2(1)	1–3(1)	–	–	–	–	
11	1,2(1)	0–2(1) <sup>b</sup>	0–2 <sup>e</sup>	1,2(1)	1	1	1	1–3(1)	–	–	–	–	
12	1–4(2)	–	–	–	–	–	–	–	–	–	–	–	
13	–	0,1 <sup>c</sup>	–	–	–	–	–	–	–	–	–	–	
14	–	–	–	1	1	1	1	1	1,2(1)	–	–	–	

<sup>a</sup>Main branches; <sup>b</sup>seta 10 of Reinert (1973); <sup>c</sup>usually represented by an empty alveolus; <sup>d</sup>normally absent; <sup>e</sup>often absent or represented by an empty alveolus.

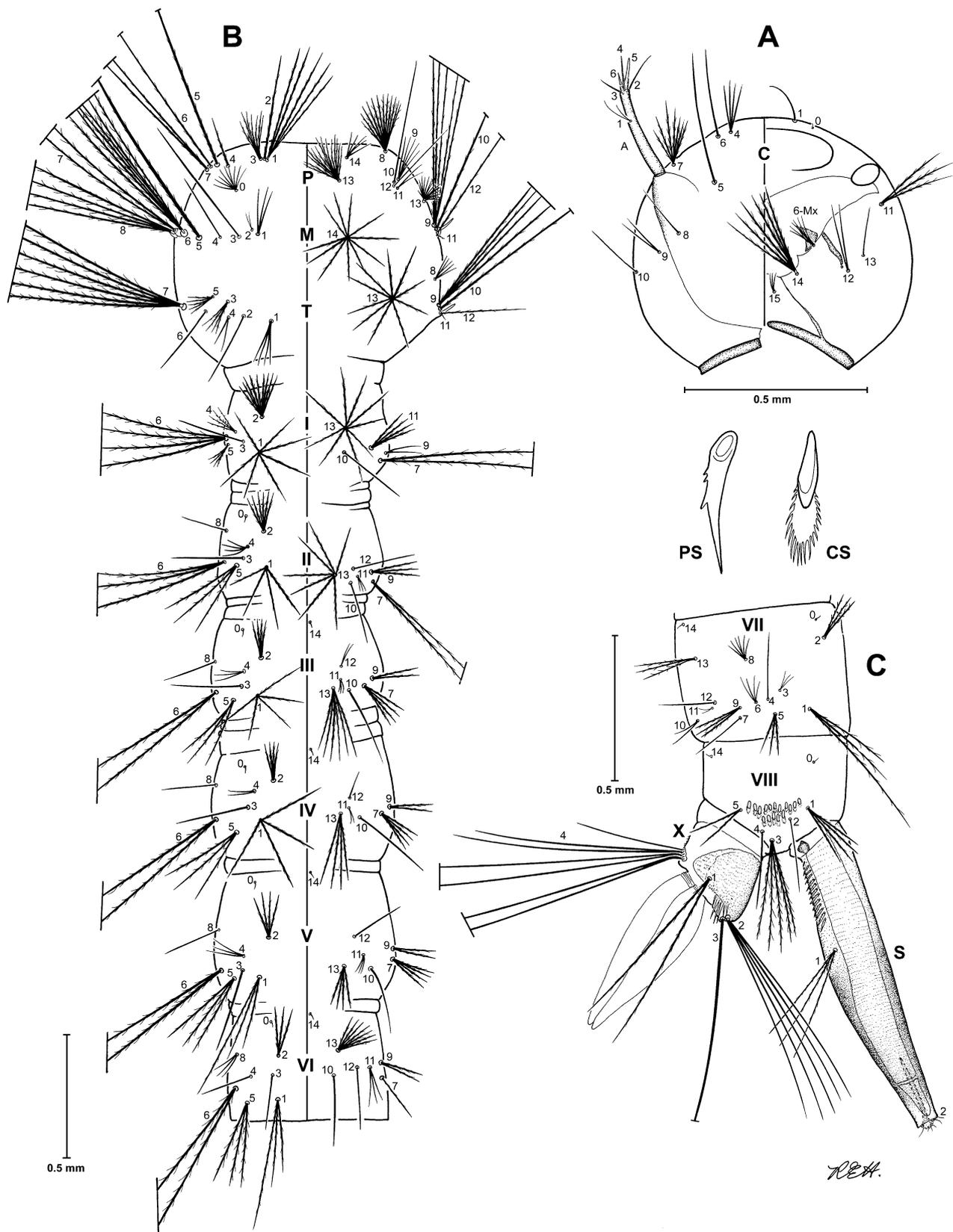


**FIGURE 1.** Pupa and male genitalia of *Bothaella manhi*: A, pupa, left side of cephalothorax, dorsal to right; B, pupa, dorsal (left) and ventral (right) aspects of metathorax and abdomen; C, male genitalia, dorsal aspect (the sigmoidal seta, arrow, is diagnostic for the species). BML, basal mesal lobe; CT, cephalothorax; Gc, gonocoxite; Gs, gonostylus; Pa, paddle; Pr, proctiger; I–VIII, abdominal segments I–VIII; 1–14, setal numbers for specified areas, e.g. seta 3-I.

**Larva, fourth-instar.** Character and positions of setae as illustrated in Fig. 2; numbers of branches in Table 2. *Head:* Round in dorsal view, length about 0.70 mm, width about 0.75 mm; moderately tanned, collar and dorsomentum more darkly tanned; median labral plate very narrow, indistinct; hypostomal suture complete to posterior tentorial pit, with short extension posteriorly to collar; dorsomentum with 10 or 11 teeth on either side of median tooth. Seta 4-C relatively long, with 4–6(4) simple branches (distinction from *Bo. alongi*); seta 9-C inserted posterior to level of 8-C; seta 13-C inserted much closer to 12-C than to 11-C; seta 14-C strongly developed, longer than labiogula, with 4 aciculate branches; seta 6-Mx stellate, with 4 aciculate branches. *Antenna:* Cylindrical, moderately tanned, surface smooth; length 0.26 mm. Seta 1-A double, simple, length about twice diameter of antenna, inserted beyond mid-length of antenna; setae 2,3-A inserted subapically. *Thorax:* Integument hyaline, smooth. Setae 0,1,3,8,13,14-P, 13-M and 5,8-T somewhat stellate (not obvious in Fig. 2), minutely spiculate; setae 14-M and 13-T distinctly stellate, minutely spiculate; setae 1–3-P not borne on a setal support plate; seta 2-P single, longer than 1,3,4-P; seta 13-P more strongly developed than 14-P, similar to 8-P; 5-T also relatively strongly developed, similar to 8-T. *Abdomen:* Setae 1,5-I–VIII, 2,13-I–VII, 9-II–VII, 4,11-I and 1,5-VIII well developed, distinctly stellate, minutely aciculate; seta 1-V–VII with one branch distinctly longer than the others; seta 2-I–VII inserted directly anterior to slightly anterolateral to seta 1; seta 3-I–VI single, moderately long, 3-VII short, double or triple, similar to 4-II–V; seta 4-VI–VIII single, similar to 3-I–VI; seta 6-I–VI long, branched, aciculate; seta 7-I,II similar to 6-I,II but with fewer branches, 7-III–VI much shorter than 7-I,II, 7-III–V usually with 4 or 5 branches (range 3–6), 7-VI single; 12-I absent; seta 9-I dissimilar to 9-II–VII, double; seta 8-II–V single, 8-VI double or triple. *Segment VIII:* Comb comprised of 16–24 evenly fringed scales in 2 irregular rows. Seta 3-VIII noticeably much longer than 1,5-VIII, reaching to or beyond most distal pecten spine. *Siphon:* Tapered and slightly bent anteriorly beyond seta 1-S; lightly tanned basal to seta 1-S, progressively darker from seta 1-S to apex; length about 1.10 mm, width at midlength about 0.17 mm, index about 6.5; acus present, small, attached to base of siphon; pecten comprised of 12–18 close-set spines with 3–5 ventral denticles on proximal half, most distal denticle largest, borne at midlength. Seta 1-S inserted distal to pecten, length about twice diameter of siphon, with 3 aciculate branches. *Segment X:* Saddle incomplete, moderately pigmented, with cluster of 6 or 7 relatively large spines on dorsoposterior margin; dorsal length about 0.25 mm; siphon/saddle index about 4.4; acus absent. Seta 1-X well developed, double, inserted on and much longer than saddle; ventral brush (seta 4-X) with 4 pairs of setae on grid (with transverse and lateral bars), anterior setae single, posterior setae double. Dorsal and ventral anal papillae equally long, longer than saddle, length about 0.5 mm, relatively thick proximally and tapered distally.

**Molecular characterization.** The dataset for the region coding for part of the COI gene comprised 658 bp from 61 individuals, and for the COII region the dataset comprised 702 bp from 59 of the same individuals (Table 3). These correspond to positions 1464–2121 and 3027–3728 of *Anopheles quadrimaculatus* Say (GenBank accession number NC\_000875), respectively. No insertions or deletions were identified and sequence alignment was unambiguous since there was no length variation in the amplified gene regions.

Among Culicidae collected in Thailand and Vietnam, the two gene regions showed similar levels of interspecific variation in uncorrected p-distance, with maximum interspecific sequence divergence of 20% between *Culex fuscocephala* Theobald and *Toxorhynchites splendens* (Wiedemann) for COI and 21% between *Mansonia bonnea* Edwards and *Uranotaenia lateralis* Ludlow for COII. The AT richness of the COI and COII fragments was 68.3 and 75.8%, respectively, in agreement with previous work and reflecting the uniquely high AT content of mitochondrial genomes of higher Diptera (Jermin & Crozier, 1994). Intraspecific uncorrected pairwise distances were less than 2% in the majority of cases, including variation within each *Bothaella* species, and there was on average 9% interspecific divergence between sequences obtained from individuals representing *Bo. helenae* and *Bo. manhi*, supporting the formal naming and description of the latter species.



**FIGURE 2.** Fourth-instar larva of *Bothaella manhi*: 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; CS, comb scale; P, prothorax; PS, pecten spine; M, mesothorax; S, siphon; T, metathorax; I–VIII and X, abdominal segments I–VIII and X; 1–15, setal numbers for specified areas, e.g. seta 5-C.

TABLE 2. Range (mode) of branches for larval setae of *Bothaella manhi* obtained from 2 larval exuviae (4 setae).

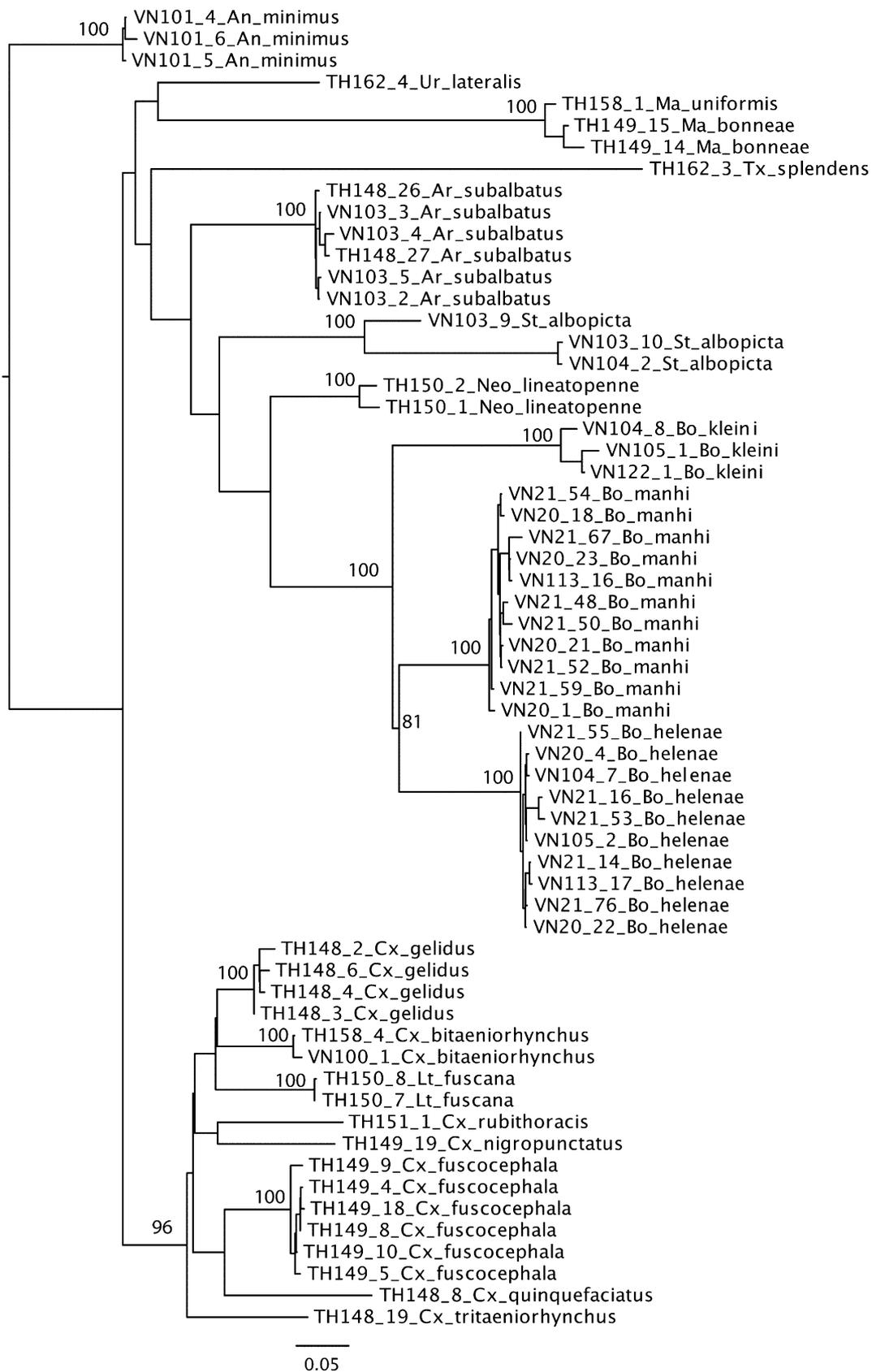
Setae no.	Abdominal segments												
	Head	Thorax			I	II	III	IV	V	VI	VII	VIII	X
0	?												
1	1	3,4	2,3	3,4	4	4	4	4	3,4(3)	2,3(3)	3	3,4(4)	2
2	-	1	2	1	5-9(5)	5,6	4,5(4)	4,5(4)	3,4(4)	3,4(3)	3	1	5
3	1	5,7	1	3,4	1	1	1	1	1	1	2,3	5	1
4	4-6(4)	1	1	2	2-4(3)	4	2,3(2)	2	2,3(3)	1	1	1	-
5	1	1	1	3-5(5)	3-5	3	3,4(3)	3	3	3	3	2	-
6	1	1	5,6	1	4	3	2	2	2	2	4,5	-	-
7	6-8	2	1	6-9(6)	2,3(2)	2	3,4(4)	4-6(5)	3-5(4)	1	1	1-A	2
8	1	10-13	6-8	5,7(5)	-	1	1	1	1	2,3	6,8	-	-
9	2	3,5	5	4,5	2	2,3(3)	2	1,2(2)	1,2	1,2(2)	3	6-Mx	4
10	1	1	1	1	1	1	1	1	1	1	1,2	-	-
11	3-7	1,2	1	1	4-6(4)	2,3	3	3,4(3)	2,3(2)	3,4(4)	1,2	1-S,	3
12	1,2	1	1	1	-	1	2,3(2)	2	1	1	1	2-S,	1
13	1	11-15	6-14	7-10	7-9(7)	5-7(7)	4-7(4)	3-5(4)	3,4(3)	7	3	-	-
14	4	3,4	8,11	-	-	-	1	1	1	1	1	1	-
15	4	-	-	-	-	-	-	-	-	-	-	-	-

**Table 3:** GenBank accession numbers and collection data for specimens included in the phylogenetic analyses.

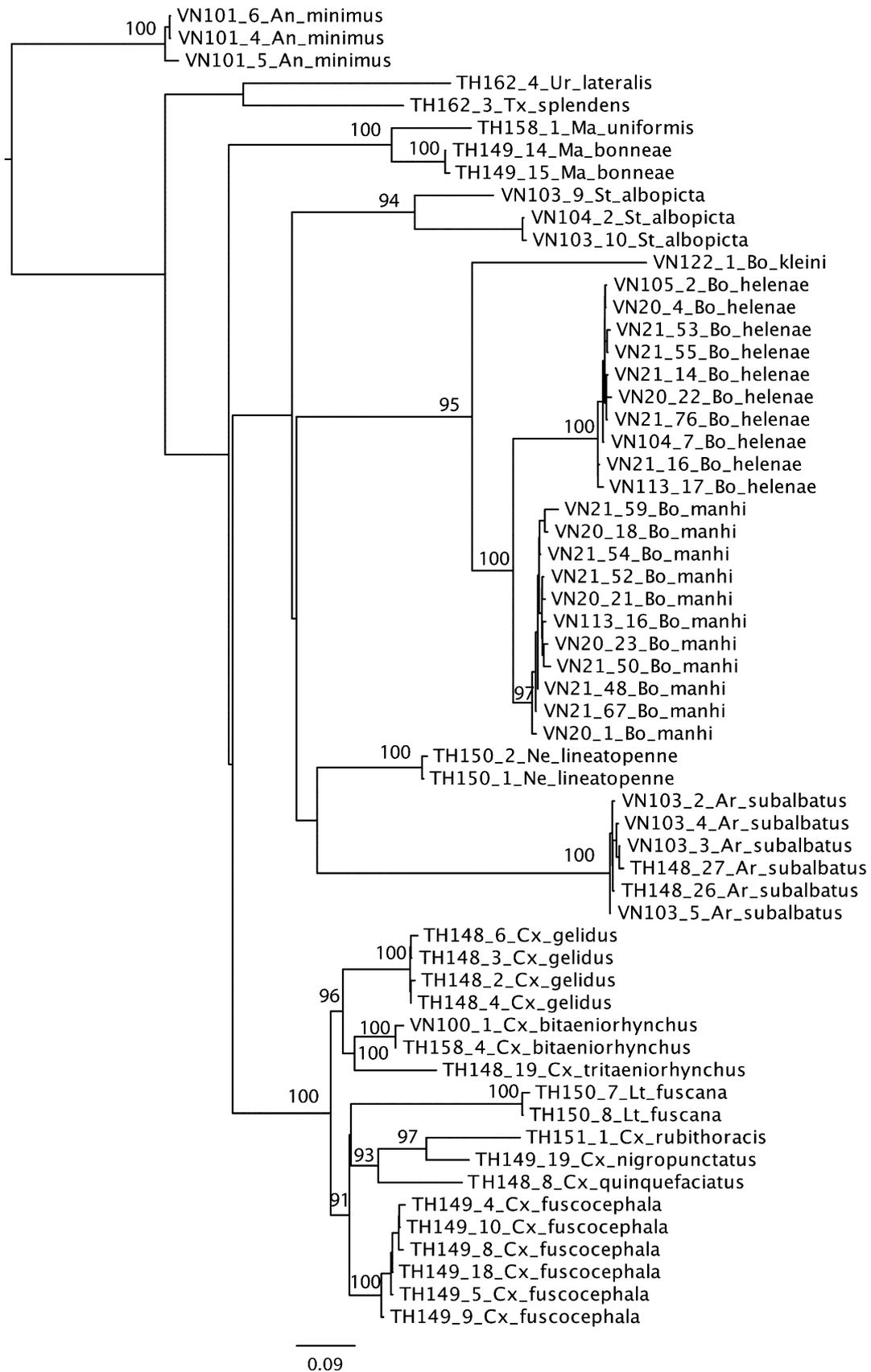
Genus	Species	Specimen number	COI accession number	COII accession number	Date collected	Latitude	Longitude	Collecting method	Province	Locality*
<i>Bothaella</i>	<i>helenae</i>	VN20-4	HQ398919	HQ398985	05/07/2000	–	–	D-IR	NB	Cuc Phuong NP
<i>Bothaella</i>	<i>helenae</i>	VN20-22	HQ398918	HQ398983	05/07/2000	–	–	D-IR	NB	Cuc Phuong NP
<i>Bothaella</i>	<i>helenae</i>	VN21-14	HQ398920	HQ398986	05/07/2000	–	–	D-IR	NB	Cuc Phuong NP
<i>Bothaella</i>	<i>helenae</i>	VN21-16	HQ398921	HQ398987	05/07/2000	–	–	D-IR	NB	Cuc Phuong NP
<i>Bothaella</i>	<i>helenae</i>	VN21-55	HQ398914	HQ398993	05/07/2000	–	–	D-IR	NB	Cuc Phuong NP
<i>Bothaella</i>	<i>helenae</i>	VN21-76	HQ398923	HQ398996	05/07/2000	–	–	D-IR	NB	Cuc Phuong NP
<i>Bothaella</i>	<i>helenae</i>	VN21-53	HQ398922	HQ398991	05/07/2000	–	–	D-IR	NB	Cuc Phuong NP
<i>Bothaella</i>	<i>helenae</i>	VN104-7	HQ398915	HQ398975	19/08/2008	N 20,14.801	E 105,42.900	BPA	NB	Cuc Phuong NP
<i>Bothaella</i>	<i>helenae</i>	VN105-2	HQ398916	HQ398976	19/08/2008	N 20,19.242	E 105,37.684	BPA	NB	Cuc Phuong NP
<i>Bothaella</i>	<i>helenae</i>	VN113-17	HQ398917	HQ398978	20/08/2008	N 20,14.753	E 105,42.934	BPA	NB	Cuc Phuong NP
<i>Bothaella</i>	<i>kleini</i>	VN104-8	HQ398913	NA	19/08/2008	N 20,14.801	E 105,42.900	BPA	NB	Cuc Phuong NP
<i>Bothaella</i>	<i>kleini</i>	VN105-1	HQ398911	NA	19/08/2008	N 20,19.242	E 105,37.684	BPA	NB	Cuc Phuong NP
<i>Bothaella</i>	<i>kleini</i>	VN122-1	HQ398912	HQ398979	20/08/2008	N 20,15.767	E 105,42.964	BPA	NB	Cuc Phuong NP
<i>Bothaella</i>	<i>manhi</i>	VN20-1	HQ398924	HQ398981	05/07/2000	–	–	D-IR	NB	Cuc Phuong NP
<i>Bothaella</i>	<i>manhi</i>	VN20-18	HQ398933	HQ398980	05/07/2000	–	–	D-IR	NB	Cuc Phuong NP
<i>Bothaella</i>	<i>manhi</i>	VN20-21	HQ398927	HQ398982	05/07/2000	–	–	D-IR	NB	Cuc Phuong NP
<i>Bothaella</i>	<i>manhi</i>	VN20-23	HQ398928	HQ398984	05/07/2000	–	–	D-IR	NB	Cuc Phuong NP
<i>Bothaella</i>	<i>manhi</i>	VN21-48	HQ398929	HQ398988	05/07/2000	–	–	D-IR	NB	Cuc Phuong NP
<i>Bothaella</i>	<i>manhi</i>	VN21-50	HQ398934	HQ398989	05/07/2000	–	–	D-IR	NB	Cuc Phuong NP
<i>Bothaella</i>	<i>manhi</i>	VN21-52	HQ398930	HQ398990	05/07/2000	–	–	D-IR	NB	Cuc Phuong NP
<i>Bothaella</i>	<i>manhi</i>	VN21-54	HQ398931	HQ398992	05/07/2000	–	–	D-IR	NB	Cuc Phuong NP
<i>Bothaella</i>	<i>manhi</i>	VN21-59	HQ398932	HQ398994	05/07/2000	–	–	D-IR	NB	Cuc Phuong NP
<i>Bothaella</i>	<i>manhi</i>	VN21-67	HQ398925	HQ398995	05/07/2000	–	–	D-IR	NB	Cuc Phuong NP
<i>Bothaella</i>	<i>manhi</i>	VN113-16	HQ398926	HQ398977	20/08/2008	N 20,14.753	E 105,42.934	BPA	NB	Cuc Phuong NP
<i>Anopheles</i>	<i>minimus</i>	VN101-4	HQ398936	HQ398965	18-19/08/2008	N 20,15.547	E 105,42.389	LT + dry ice	NB	Cuc Phuong NP
<i>Anopheles</i>	<i>minimus</i>	VN101-5	HQ398935	HQ398966	18-19/08/2008	N 20,15.547	E 105,42.389	LT + dry ice	NB	Cuc Phuong NP
<i>Anopheles</i>	<i>minimus</i>	VN101-6	HQ398937	HQ398967	18-19/08/2008	N 20,15.547	E 105,42.389	LT + dry ice	NB	Cuc Phuong NP
<i>Armigeres</i>	<i>subalbatus</i>	VN103-2	HQ398904	HQ398969	19/08/2008	N 20,14.753	E 105,42.935	BPA	NB	Cuc Phuong NP
<i>Armigeres</i>	<i>subalbatus</i>	VN103-3	HQ398905	HQ398970	19/08/2008	N 20,14.753	E 105,42.935	BPA	NB	Cuc Phuong NP
<i>Armigeres</i>	<i>subalbatus</i>	VN103-4	HQ398906	HQ398971	19/08/2008	N 20,14.753	E 105,42.935	BPA	NB	Cuc Phuong NP
<i>Armigeres</i>	<i>subalbatus</i>	VN103-5	HQ398907	HQ398972	19/08/2008	N 20,14.753	E 105,42.935	BPA	NB	Cuc Phuong NP
<i>Armigeres</i>	<i>subalbatus</i>	TH148-26	HQ398908	HQ398939	13/08/2008	N 18,45.058	E 098,56.377	BPA	CM	Mae Hae
<i>Armigeres</i>	<i>subalbatus</i>	TH148-27	HQ398903	HQ398940	13/08/2008	N 18,45.058	E 098,56.377	BPA	CM	Mae Hae
<i>Culex</i>	<i>bitaeniorhynchus</i>	VN100-1	HQ398899	HQ398964	18-19/08/2008	N 20,15.385	E 105,42.539	LT + dry ice	NB	Cuc Phuong NP
<i>Culex</i>	<i>bitaeniorhynchus</i>	TH158-4	HQ398889	HQ398964	14/08/2008	N 18,49.979	E 099,01.795	LT + dry ice	CM	Amphoe San Sai
<i>Culex</i>	<i>fuscoccephala</i>	TH149-4	HQ398889	HQ398951	13/08/2008	N 18,45.058	E 098,56.377	BPA	CM	Mae Hae
<i>Culex</i>	<i>fuscoccephala</i>	TH149-5	HQ398891	HQ398952	13/08/2008	N 18,45.058	E 098,56.377	BPA	CM	Mae Hae
<i>Culex</i>	<i>fuscoccephala</i>	TH149-8	HQ398890	HQ398953	13/08/2008	N 18,45.058	E 098,56.377	BPA	CM	Mae Hae
<i>Culex</i>	<i>fuscoccephala</i>	TH149-9	HQ398954	13/08/08	13/08/2008	N 18,45.058	E 098,56.377	BPA	CM	Mae Hae
<i>Culex</i>	<i>fuscoccephala</i>	TH149-10	HQ398887	HQ398946	13/08/2008	N 18,45.058	E 098,56.377	BPA	CM	Mae Hae
<i>Culex</i>	<i>fuscoccephala</i>	TH149-18	HQ398888	HQ398949	13/08/2008	N 18,45.058	E 098,56.377	BPA	CM	Mae Hae
<i>Culex</i>	<i>gelidus</i>	TH148-2	HQ398894	HQ398941	13/08/2008	N 18,45.058	E 098,56.377	BPA	CM	Mae Hae
<i>Culex</i>	<i>gelidus</i>	TH148-3	HQ398895	HQ398942	13/08/2008	N 18,45.058	E 098,56.377	BPA	CM	Mae Hae
<i>Culex</i>	<i>gelidus</i>	TH148-4	HQ398892	HQ398943	13/08/2008	N 18,45.058	E 098,56.377	BPA	CM	Mae Hae
<i>Culex</i>	<i>gelidus</i>	TH148-6	HQ398893	HQ398944	13/08/2008	N 18,45.058	E 098,56.377	BPA	CM	Mae Hae
<i>Culex</i>	<i>nigropunctatus</i>	TH149-19	HQ398882	HQ398950	13/08/2008	N 18,45.058	E 098,56.377	BPA	CM	Mae Hae
<i>Culex</i>	<i>quinquefasciatus</i>	TH148-8	HQ398883	HQ398945	13/08/2008	N 18,45.058	E 098,56.377	BPA	CM	Mae Hae
<i>Culex</i>	<i>rubithoracis</i>	TH151-1	HQ398884	HQ398959	14/08/2008	N 18,41.963	E 099,08.724	BPA	L	Amphoe Ban Thi
<i>Culex</i>	<i>tritaeniorhynchus</i>	TH148-19	HQ398885	HQ398938	13/08/2008	N 18,45.058	E 098,56.377	BPA	CM	Mae Hae
<i>Lutzia</i>	<i>fuscana</i>	TH150-7	HQ398896	HQ398957	13/08/2008	N 18,44.808	E 098,57.003	BPA	CM	Mae Hae
<i>Lutzia</i>	<i>fuscana</i>	TH150-8	HQ398897	HQ398958	13/08/2008	N 18,44.808	E 098,57.003	BPA	CM	Mae Hae
<i>Mansonia</i>	<i>bonneae</i>	TH149-14	HQ398878	HQ398947	13/08/2008	N 18,45.058	E 098,56.377	BPA	CM	Mae Hae
<i>Mansonia</i>	<i>bonneae</i>	TH149-15	HQ398879	HQ398948	13/08/2008	N 18,45.058	E 098,56.377	BPA	CM	Mae Hae
<i>Mansonia</i>	<i>uniformis</i>	TH158-1	HQ398880	HQ398960	14/08/2008	N 18,49.979	E 099,01.795	LT + dry ice	CM	Amphoe San Sai
<i>Neomelanicolonia</i>	<i>lineatopempe</i>	TH150-1	HQ398909	HQ398955	13/08/2008	N 18,44.808	E 098,57.003	BPA	CM	Mae Hae
<i>Neomelanicolonia</i>	<i>lineatopempe</i>	TH150-2	HQ398910	HQ398956	13/08/2008	N 18,44.808	E 098,57.003	BPA	CM	Mae Hae
<i>Stegomyia</i>	<i>albopicta</i>	VN103-9	HQ398902	HQ398973	19/08/2008	N 20,14.753	E 105,42.935	BPA	NB	Cuc Phuong NP
<i>Stegomyia</i>	<i>albopicta</i>	VN103-10	HQ398900	HQ398968	19/08/2008	N 20,14.753	E 105,42.935	BPA	NB	Cuc Phuong NP
<i>Stegomyia</i>	<i>albopicta</i>	VN104-2	HQ398901	HQ398974	19/08/2008	N 20,14.801	E 105,42.900	BPA	NB	Cuc Phuong NP
<i>Toxorhynchites</i>	<i>splendens</i>	TH162-3	HQ398877	HQ398962	16/08/2008	N 18,54.087	E 098,49.821	BPA	CM	Amphoe San Sai
<i>Uranotaenia</i>	<i>lateralis</i>	TH162-4	HQ398881	HQ398963	16/08/2008	N 18,54.087	E 098,49.821	BPA	CM	Amphoe San Sai

BPA = Backpack aspirator; CM = Chiang Mai; D-IR = Dropper – Individual rearing of larva or pupa; L = Lamphun; LT = Light trap; NB = Ninh Binh; NP = National Park; TH = Thailand; VN = Vietnam.

The maximum likelihood trees for COI and COII are shown in Figs 3 and 4, respectively. In the case of both trees, all species-level clades were supported by high (100%) posterior probabilities. The only exception comprised two specimens representing *Ma. bonneae* with posterior probabilities of 75% in the phylogeny based on the COI region but 100% in the phylogeny based on the COII region. Importantly, in some cases, sequences for a given species originate from wild mosquitoes from widely dispersed geographic locations, namely the *Armigeres subalbatus* (Coquillett) and *Culex bitaeniorhynchus* Giles lineages, or from different sampling years, for example 2000 and 2008 within both *Bo. manhi* and *Bo. helenae*, but despite this, sequences still group strongly according to species. In addition, currently accepted species groupings are well resolved in ML phylogenies of both the COI and COII regions. Taken together, the results strongly support (1) the utility of mtDNA for species-level identification of Culicidae and (2) the formal naming and description of *Bo. manhi* and its inferred sister relationship to *Bo. helenae*, with *Bo. kleini* forming their near relative. Interestingly, in contrast, phylogenetic structure at the subfamily level for both genes was either absent or of limited support, reflected by posterior probabilities of less than 50% in general and in agreement with previous studies (Cook *et al.*, 2005).



**FIGURE 3.** Bayesian phylogeny of the “barcode” region of the COI nucleotide dataset of the Culicidae collected in Vietnam and Thailand. Posterior probabilities (percent) of 80 or higher are shown for main clades only. See Table 3 for specimen collection data and accession numbers. All horizontal branch lengths are drawn to a scale of substitutions per site. The tree is rooted on *Anopheles minimus* Theobald.



**FIGURE 4.** Bayesian phylogeny of the COII nucleotide dataset of the Culicidae collected in Vietnam and Thailand. Posterior probabilities (percent) of 80 or higher are shown for main clades only. See Table 3 for specimen collection data and accession numbers. All horizontal branch lengths are drawn to a scale of substitutions per site. The tree is rooted on *An. minimus*.

**Systematics.** Diagnostic and differential features that distinguish the three species of *Bothaella* with banded hindtarsi that are known to occur in Vietnam are listed in Table 4. In view of these differences, and characters that distinguish the five species of *Bothaella*, *Bo. manhi* is most similar to *Bo. helenae*, the adult females and pupae of which are inseparable. This is also supported by ML phylogenetic analyses of DNA sequences, which suggests that *Bo. manhi* + *Bo. helenae* forms a sister group to *Bo. kleini* (Figs 3 and 4).

**Bionomics.** The immature stages of *Bo. manhi* were collected from very small holes in limestone outcrops in rainforest. The holes contained clear fresh water and were heavily shaded. Adult females were collected resting on vegetation in forest and a primate sanctuary. Indirect evidence suggests that females may feed on primates and other mammals. Nothing else is known about the bionomics of the species.

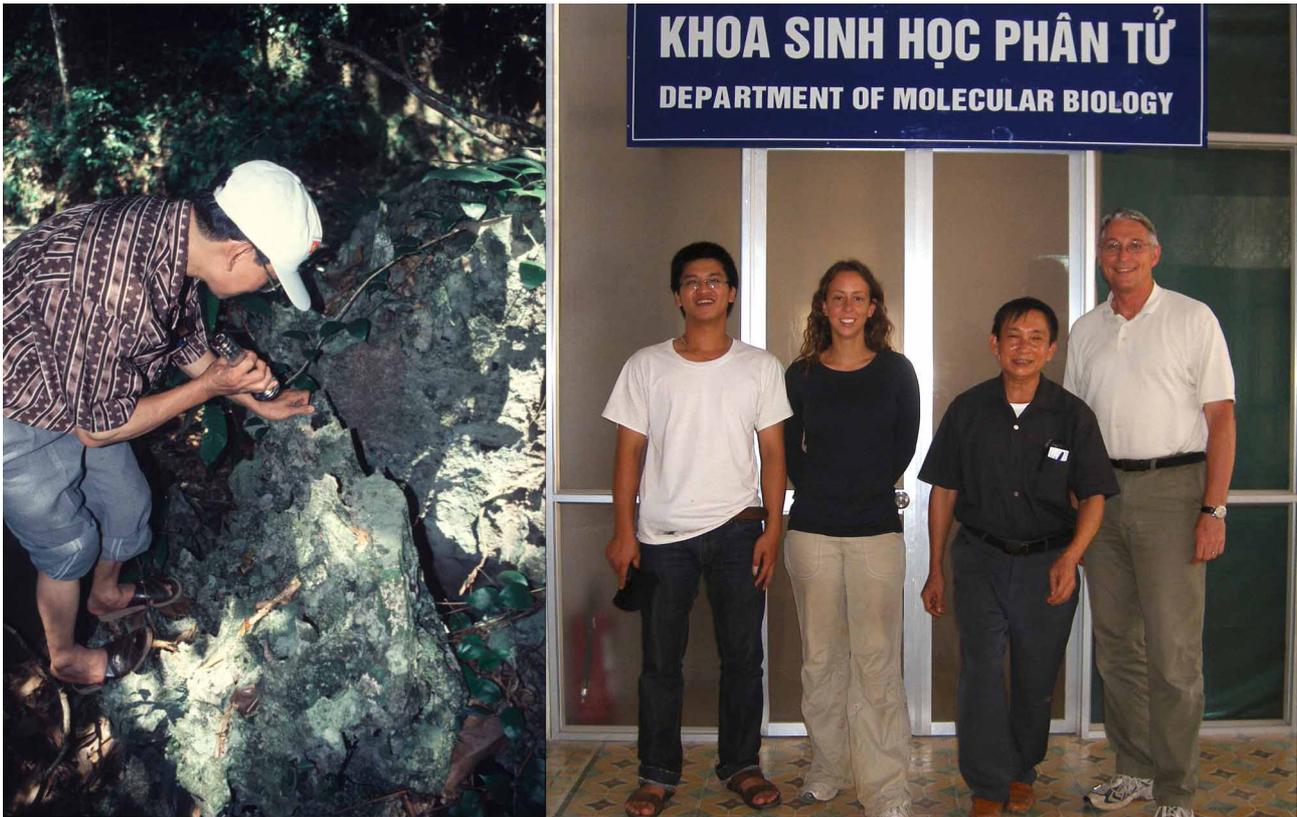
**TABLE 4.** Comparison of diagnostic and differential characters observed in three species of *Bothaella* with banded hindtarsi that occur in northern Vietnam. *Bothaella kleini* also occurs in northern Vietnam but its hindtarsi are entirely dark-scaled.

	Character	<i>Bo. alongi</i> <sup>1</sup>	<i>Bo. helenae</i> <sup>2</sup>	<i>Bo. manhi</i>
Adults	Postgena	Dark-scaled	With silvery scale-patch	With silvery scale-patch
	Postpronotal setae	3	3 or 4	2 or 3
	Upper mesokatepisternal setae	2 or 3	3	2 or 3
	Forecoxa	Dark-scaled anteriorly at apex	Patch of silvery scales anteriorly at apex	Patch of silvery scales anteriorly at apex
	Hindfemur	Proximal 0.70 pale-scaled with dorsal line of dark scales	Proximal 0.75–0.80 pale-scaled with dorsal line of dark scales	Proximal 0.50–0.60 pale-scaled, usually without trace of dorsal dark scales
	Wing vein R2+3	Longer than vein R2	Shorter than vein R2	Shorter than vein R2
	Basal mesal lobe (♂ genitalia)	Unknown	Without long sigmoid seta	With long sigmoid seta
Larvae	Seta 4-C	10–14 branches	4–8(4) branches	4–6(4) branches
	Seta 14-C	5–9 branches	5–8(6) branches	4 branches
	Seta 6-Mx	6–11 branches	9–15 branches	4 branches
	Seta 0-P	15,16 branches	8–14(9)	5,6 branches
	Seta 3-P	15–17 branches	11–15(12) branches	5,7 branches
	Seta 1-M	Multi-branched	5–7(6) branches	2,3 branches
	Seta 1-T	22–24 branches	5–12(6)	3,4 branches
	Seta 8-T	11 branches	10–16(11) branches	4–6(4) branches
	Seta 1-II–IV	Multi-branched	6–10 branches	4 branches
	Seta 6-I	7–9 branches	3,4(3) branches	4 branches
	Seta 6-II	7,8 branches	3 branches	3 branches
	Seta 4-III	Stellate	5–8(5) branches	2,3(3) branches
	Seta 4-IV	Stellate	4–9(6) branches	2 branches
	Seta 9-III–VI	?	3–6(3,4) branches	1,2(2) branches
	Seta 5-IV–VII	Stellate	4,5(4) branches	3 branches
	Seta 1-VIII	14–17 branches	8–11(8) branches	3,4(4) branches
	Seta 5-VIII	6 branches	3–7(5) branches	2 branches
	Pecten spines	9–11	13–16	12–18
	Siphon (index)	Short (2.2)	Long (ca. 6.5)	Long (4.1–4.9)

<sup>1</sup>Data from Reinert & Harbach (2006); <sup>2</sup>data from Reinert (1973).

**Distribution.** *Bothaella manhi* is only known from the Cuc Phuong National Park of Ninh Binh Province in northern Vietnam.

**Etymology.** *Bothaella manhi* is dedicated to the memory of Dr Nguyen Duc Manh, our respected colleague, co-researcher and friend who died unexpectedly while this manuscript was in preparation. Dr Manh was Head of the Department of Molecular Biology, National Institute of Malariology, Parasitology and Entomology (NIMPE) in Hanoi prior to his death on 7 July 2010. He will be remembered for his exuberant interest and participation in entomological field studies and his contributions to mosquito taxonomy. Dr Manh (Fig. 5) arranged and participated in the visits to the Cuc Phuong National Park that resulted in the discovery of the new species named in his honour here. (NB: In Vietnam, as in many Asian countries, the family name is placed before the individual's given name; hence, individuals are addressed by their given name.)



**FIGURE 5.** Left: Dr Nguyen Duc Manh examining a rock-hole habitat of *Bothaella* larvae during mosquito surveys conducted in the Cuc Phuong National Park in July 2000. Right: Dr Manh (second from right) with field assistant Nguyen Hong Hanh (far left), Shelley Cook and Ralph Harbach outside the Molecular Biology Laboratory of the National Institute of Malariology, Parasitology and Entomology prior to field studies conducted in the same park in August 2008.

**Type series.** Forty-six specimens: 10 ♀, 4 ♂ genitalia, 8 ♂, 8 ♂ genitalia, 2 Le, 14 Pe. *Holotype*, ♂ (VN21-49), with Pe and dissected genitalia on microscope slides, VIETNAM: *Ninh Binh Province*, Cuc Phuong National Park, rock hole, 5 July 2000 (Harbach *et al.*). *Paratypes* (same locality and collectors as holotype), 2 ♀LePe (VN20-23, VN21-51); 8 ♀Pe (VN20-1, -2 with dissected genitalia on microscope slides; VN21-48, -50, -67; VN21-59 with dissected genitalia on microscope slide; VN47-2); 1 ♀ (VN47-2); 7 ♂Pe (VN20-16, -18, -19, -21 and VN21-49, -52, -54 with dissected genitalia on microscope slides); 1 ♀ (VN115-2), same locality, forest edge near stream, and 1 ♂ (VN113-16), Endangered Primate Sanctuary, both with dissected genitalia on microscope slides, 20 August 2008 (Cook *et al.*). Specimens for which DNA sequence are available are indicated in Table 3. The type series is deposited in the Natural History Museum (BMNH), London.

## Acknowledgements

We are grateful to field workers from the National Institute of Malariology, Parasitology and Entomology for their participation in the field work; to Luis Hernandez, Department of Entomology, Natural History Museum (NHM), London, for taking the photomicrographs used to prepare Fig. 1C. The work in Vietnam and Thailand was conducted as part of a Sir Henry Wellcome Trust Fellowship (Grant number 082744) awarded to Shelley Cook for her study entitled “Emerging arboviruses: the effect of vector and host biology and genetic diversity on the emergence and maintenance of the flaviviruses”. The field work was also supported by funds provided from the Department of Entomology Investment Fund (NHM, London).

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