Acta Tropica 215 (2021) 105799

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Molecular species delimitation reveals high diversity in the mosquito *Anopheles tessellatus* Theobald, 1901 (Diptera, Culicidae) across its range

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ARTICLE INFO

Keywords:
Vector
Mosquito
Asia
Malaria
Filariasis
Species delimitation
Tessellatus complex

ABSTRACT

Anopheles tessellatus is a potentially important vector found across South, East and Southeast Asia. While it was formerly considered a formidable vector of human *Plasmodium* and filarial parasites in the Maldives, and of lesser importance as a vector of human *Plasmodium* in Sri Lanka and parts of Indonesia, it is currently of little or unknown health importance in many other parts of its range. This study describes the genetic diversity and evolutionary relationships among *An. tessellatus* populations in nine Asian countries at the COI gene using maximum-likelihood and Bayesian phylogenetic inference tree and cluster-based species delimitation approaches. These analyses reveal exceptional levels of genetic diversity in *An. tessellatus* populations across its known range, and identify up to six putative species in the newly determined Tessellatus Complex. The existence of such cryptic diversity has potentially important consequences for vector management and disease control. Differences in the ecologies and life histories among these species may have considerable impact on vectorial capacity and may go some way towards explaining why *An. tessellatus* s.l. has such varying degrees of public health importance across its range.

1. Introduction

Anopheles tessellatus Theobald, 1901 was originally described from Taiping, Perak in northwest peninsular Malaysia, and is the sole representative of the morphologically distinct Tessellatus Group in the Neomyzomyia Series of the *Anopheles* subgenus *Cellia* (Harbach, 2004). However, the taxonomic history of the species is somewhat complex, indicating morphological diversity between populations, which are captured by its numerous synonyms and subspecies. Three valid subspecies are recognized – the nominate *An. tessellatus tessellatus* Theobald, 1901, *An. tessellatus kalawara* Stoker & Waktoedi Koesoemawinangoen, 1949, described from Indonesia, and *An. tessellatus orientalis* (Swellengrebel & Swellengrebel de Graaf, 1920) described from Sulawesi, Java, and the Moluccas – along with six valid synonyms (Wilkerson et al., 2020). Three synonyms exist from Taiwan (*formosae* Hatori 1901, *kinoshitai* Koidzumi, 1917, and *taiwanensis* Koidzumi 1917), and a further one each from Sumatra (*deceptor* Dönitz, 1902), Mindanao, Philippines (*thorntonii* Ludlow, 1904) and Sri Lanka (*ceylonica* Newstead and Carter, 1910), respectively. The species appears widespread, occurring from the Indian subcontinent (Pakistan, India, Sri Lanka, Maldives, Nepal, Bhutan, Bangladesh) through mainland Southeast Asia (People's Republic of China, Myanmar, Malaysia, Guam, Thailand, Cambodia, Laos, Vietnam), and on the islands of Taiwan, Japan (Yonagunijima), The Philippines, Indonesia, East Timor, Papua New Guinea, the Solomon Islands and Guam (Fig. 1) (Wilkerson et al., 2020).

In Cambodia (St Laurent et al., 2017, 2016), The Philippines (Aure et al., 2016; Walker et al., 1998), Sri Lanka (Amerasinghe et al., 1999), Taiwan (Chang et al., 2008), and Thailand (Rattanarithikul et al., 1996), *Anopheles tessellatus* is regarded as predominantly zoophilic, feeding mainly outdoors on bovids. However, in bloodmeal studies, Human Blood Indices (HBI; proportion found positive for human blood) of 5–10% have been reported in Sri Lanka (Amerasinghe et al., 1999), and Indonesia (Elyazar et al., 2013). The species also exhibits multiple blood feeding (Ramasamy et al., 2000) and mixed blood meals that include human blood have been found in Malaysia (Sandosham, 1959) and Sri Lanka (Amerasinghe et al., 1999). These feeding habits, coupled with endophagy in some coastal locations of Java, Indonesia (Stoops et al., 2009), are likely to increase human-vector contact and the risk of zoonotic infection.

Despite its dominantly zoophilic feeding habits, *An. tessellatus* has

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https://doi.org/10.1016/j.actatropica.2020.105799
Received 30 October 2020; Received in revised form 11 December 2020; Accepted 15 December 2020
Available online 23 December 2020

been incriminated in the transmission of several important human pathogens. Laboratory studies established the vector competency of *Anopheles tessellatus* to *Plasmodium vivax* and *P. falciparum* (Gamage--Mendis et al., 1993) and Japanese Encephalitis virus (Banerjee et al., 1977). Furthermore, the species has been found naturally infected with *P. falciparum* in Sumatra, Indonesia (Elyazar et al., 2013), *P. vivax* and *P. falciparum* in Sri Lanka (Amerasinghe et al., 1991), and with Japanese Encephalitis virus in Taiwan (Su et al., 2014). *Anopheles tessellatus* is a major field vector of filariasis caused by *Wuchereria bancrofti* in the Maldives (Iyengar, 1952), and has been found naturally infected with the flavivirus Kampung Karu virus (a close relative of dengue virus; Guzman et al., 2018) in Malaysian Borneo (Young et al., 2017) and Chittoor virus (a strain of Batai virus, *Bunyaviridae*) of as yet unknown public health importance in India (Singh and Pavri, 1966).

Given the complex taxonomic history, considerable geographic distribution and variable medical importance of *An. tessellatus*, it seems likely that molecular approaches may uncover hidden biodiversity within this quietly important vector. However, to date, molecular analysis of *An. tessellatus* has been limited only to DNA barcoding studies in efforts to establish national species inventories (Chan et al., 2014; Taira et al., 2012; Wang et al., 2012; Weeraratne et al., 2017). Herein, we pool currently available DNA barcode sequences (658 bp of the mitochondrial cytochrome c oxidase I gene) from those above studies (n = 28) to our own geographically diverse samples collected over many years (n = 67), to explore genetic diversity within *An. tessellatus* using Bayesian phylogenetic analysis (Ronquist and Huelsenbeck, 2003) and distance-based (Puillandre et al., 2012; Ratnasingham & Hebert, 2013) and tree-based (Kapli et al., 2017) species delimitation approaches.

2. Methods

2.1. Mosquito collection

A total of 67 *An. tessellatus* specimens were collected from Laos, Northwest Borneo (West Sarawak, Malaysia), Southeast Borneo (East Kalimantan, Indonesia), The Philippines (Luzon & Mindanao) and Vietnam by Y-ML and her colleagues and students (see acknowledgements), over the course of many field collections undertaken in south-east Asia from 1999 through 2005. Samples were largely collected as adults in cow- or caribou-baited traps. Specimens were individually stored in pierced individual Beem® capsules, and dried and stored in sealed plastic bags containing silica gel. Samples were identified using appropriate morphological keys (Rattanarithikul and Harrison, 1973; Reid, 1968).

2.2. DNA extraction & barcoding

DNA was extracted from single mosquitoes manually using the QIAgen® Dneasy Blood & Tissue Kit. Mitochondrial *cytochrome c oxidase* I (*COI*) gene sequences were amplified and directly sequenced using either the LCO1490 / HCO2198 primers (658bp) of Folmer et al. (1994) and the standard protocol of the Mosquito Barcoding Initiative

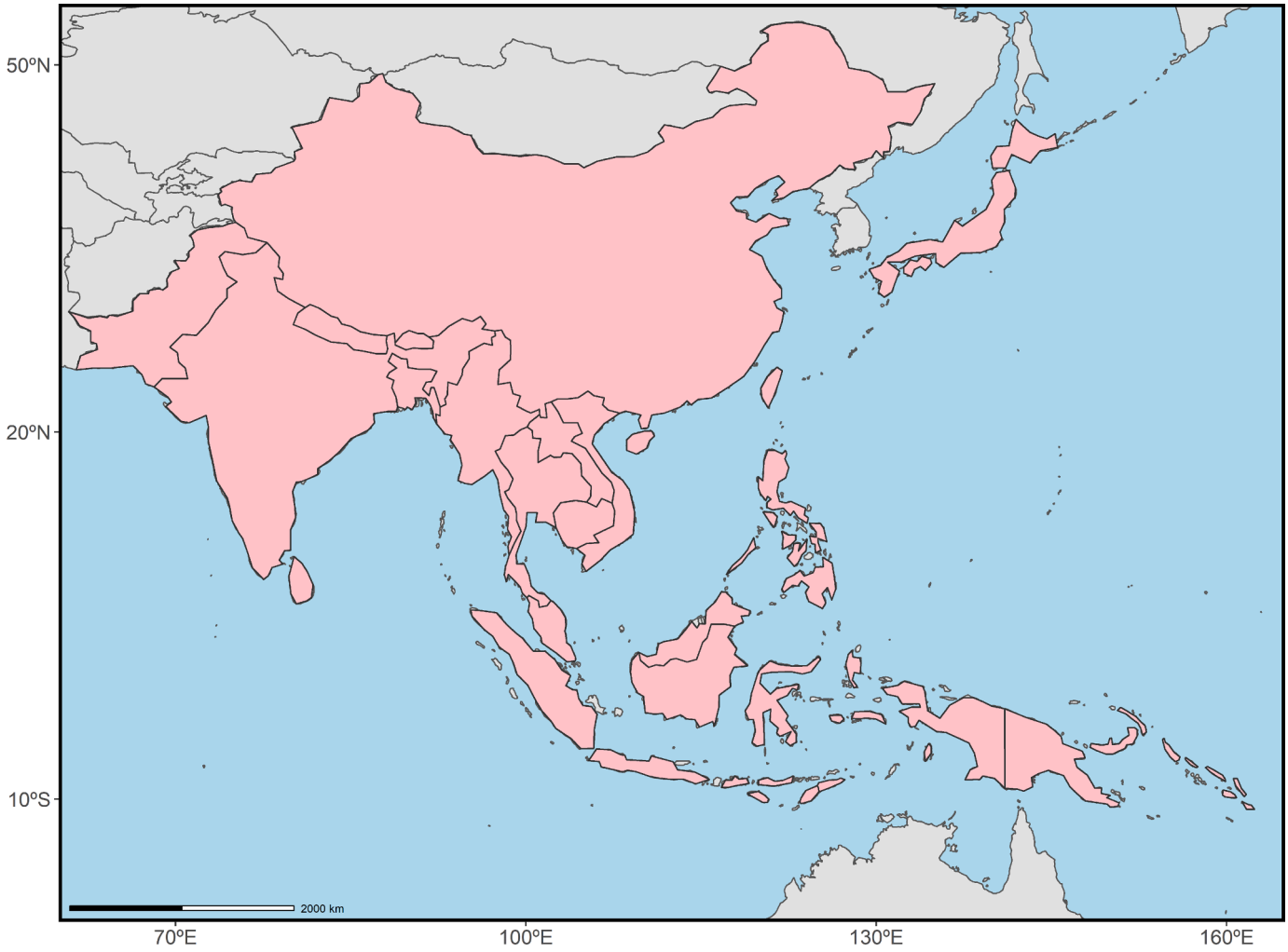

Fig. 1. Documented country-level distribution reports for *Anopheles tessellatus* s.l.

as explicitly documented in Linton et al. (2013), or a shorter amplicon (522 bp) using a combination of insect mitochondrial primers (UBC6 & UBC9) originally developed by the University of British Colombia, that correspond to C1-J-1718 and C1-N-2191, as in Simon et al. (1994). Bi-directional sequencing was carried out at the Natural History Museum, London, England (NHMUK) on an ABI 3730 automated sequencer (PE Applied BioSystems, Warrington, England) using the original PCR primers and the Big Dye Terminator Kit® (PE Applied BioSystems). Sequences were edited in Sequencher v5.4.6 (Genes Codes Corporation, Ann Arbor, MI). Similarities with publicly available sequences were assessed using BLAST (Basic Local Alignment Search Tool, http://blast.ncbi.nlm.nih.gov) and comparisons with unpublished barcode records were checked through the IDS (Identification System) of the Barcode of Life Database (BOLD, http://www.boldsystems.org). Our collected specimens yielded 67 *An. tessellatus* COI sequences. Additional *An. tessellatus* s.l. COI sequences (n = 28) were garnered from GenBank (www.ncbi.nlm.nih.gov/genbank), to include the following entries from China (JQ728050–JQ728054, JQ728102–JQ728103, MF179266–MF179271, MK685249, MK685260), Indonesia (MT753038), Japan (AB738146–AB738147), Malaysia (MT669953–MT669955), Singapore (KF564696–KF564699) and Sri Lanka (KX668149–KX668151). A total of 95 *An. tessellatus* COI were therefore included in the analyses. *Chagasia bonneae* (KF671010) and *Anopheles* (*Nyssorhynchus*) *albitarsis* sensu stricto (JQ615211) were included to serve as outgroup taxa in phylogenetic tree construction. The COI gene sequences were aligned first by nucleotides using the Muscle algorithm (Edgar, 2004) implemented in SeaView (Gouy et al., 2010), and then by amino acid using TranslatorX (Abascal et al., 2010).

2.3. Voucher specimens

COI barcode sequences, together with the original chromatograms and specimen collection details can be assessed through the Barcode of Life Database (BOLD: www.boldsystems.org) under the project TESS (*Anopheles tessellatus* –a species complex). DNA sequences (n = 67) generated in this study are available in GenBank under accession numbers MT256975–MT257041. Where specimens were collected as immatures, associated skins are deposited in the Entomology Collections of the Natural History Museum in London, England (NHMUK); specimens collected as adults are DNA vouchered only, and exemplar DNA vouchers are held in the in the frozen repository at NHMUK.

2.4. Phylogenetic analysis

Bayesian phylogenetic analysis was performed on sequences using MrBayes 3.2 (Ronquist and Huelsenbeck, 2003). Optimal evolutionary models and partitioning schemes were determined for sequence alignment using the ModelFinder (Kalyaanamoorthy et al., 2017) implemented in IQ-Tree (Nguyen et al., 2014). With the exception of 4 singletons, codon position 2 was invariable and so was excluded from phylogenetic analyses. The optimal models for codon positions 1 and 3 in ingroup sequences, testing only models supported by MrBayes (-mset mrbayes), were K2P and HKY+G, respectively. The Bayesian phylogenetic analysis consisted of two simultaneous runs, each of which were run with four Metropolis-coupled Markov chain Monte Carlo for 50 million steps, with a relative burn-in fraction of 25%. Adequate mixing was achieved by setting the chain temperature to 0.05. Convergence was reached when the average standard deviation of split frequencies between the two simultaneous runs dropped to 0.01, and the potential scale reduction factor values were all approximately 1.0 in the post-burn-in samples. A consensus tree was constructed containing nodes with posterior probability support of at least 70% using SumTrees (Sukumaran and Holder, 2015, 2010)

2.5. Species delimitation

Three species delimitation methods were employed in this analysis: Automatic Barcode Gap Discovery (ABGD), Refined Single Linkage (RESL) and multi-rate Poisson tree processes (mPTP). ABGD and RESL are both distance-based methods. ABGD assigns sequences into potential species based on the detection of a barcoding gap, interspecific variation > intraspecific variation. It does not require *a priori* species designation, but does require an intraspecific threshold (P), below which interspecific diversity will not be detected. In the current analysis, this was set to Pmin = 0.005 and Pmax = 0.1. RESL clusters sequences into operational taxonomic units (OTUs) using a graph analytical approach (Ratnasingham and Hebert, 2013). Analysis was based on a p-distance matrix using the BOLD aligner, implemented on the Barcode of Life Data (BOLD, www.barcodinglife.org) Data System v4 (Ratnasingham and Hebert, 2007). mPTP is a tree-based or "phylogeny-aware" method that uses differences in mutation rate in a phylogenetic tree to resolve interspecific and intraspecific diversity. A maximum likelihood tree was inferred from IQ-Tree (Nguyen et al., 2014) using optimal models (Codon position 1 = TNe; Codon position 3 = TIM+I) selected from the full range of models supported in ModelFinder. This tree was then used as the binary tree input for mPTP delimitation. Minimum branch length was estimated (–minbr_auto) to control for enforced non-zero branch lengths. MCMC was used to assess confidence, and 4 independent runs were conducted per analysis, set at 10 million steps, with a burn-in fraction of 10%. Convergence was reached when average standard deviation of delimitation support values fell below 0.01. Pairwise Kimura-two-parameter (K2P) distances were calculated using the Analyses of Phylogenetics and Evolution (APE) package (Paradis et al., 2004). Minimum inter-specific and maximum intra-specific distances were calculated in SPIDER (Brown et al., 2012).

3. Results

In total, 95 samples were analyzed in this study: 67 COI barcode sequences generated herein, and 28 sequences publicly available in GenBank. These sequences represent populations from at least 21 localities in nine South, Southeast and East Asian countries, and are geographically coded as in Table 1. The analyzed COI sequences ranged from 414 bp (Sri Lanka only) to 658 bp in length, with only twelve sequences at <500 bp.

3.1. Phylogenetic analysis

A basal split among the *An. tessellatus* s.l. sequences (100% Bayesian posterior probability, BPP) resolves a clade (93% BPP) comprised of Southeast Borneo (east Kalimantan; BO-KAL), Northeast and Northwest Borneo (Sarawak; BO-SAB and BO-SAR), Mindanao island in the southern Philippines (PH-MIN) and Singapore (SING) sequences, from another clade (99% BPP) comprising all mainland Asia (CHN, LAOS, VIET), Southeast Borneo (BO-KAL), Northwest Borneo (BO-SAR), Japan (JPN), Luzon (PH-LUZ), Sri Lanka (SRIL) and Sulawesi (SUL).

Within the first basal clade, there are two further moderately to strongly supported clades (>85% BPP). The first is comprised of *An. tessellatus* sequences from Philippines (Mindanao) (96% BPP), while the second (85% BPP) is made up of Singapore and Borneo (Northeast, Northwest and Southeast Borneo) sequences, each of which form sister clades (>90% BPP) with one another.

Within the second basal clade, there are two further strongly supported clades. The first of these (100% BPP) is comprised of sequences generated from samples collected in Northwest and Southeast Borneo, the island of Sulawesi and the northerly Philippine island of Luzon (PH-LUZ) sequences. The second clade (99% BPP) is comprised only of haplotypes from mainland Asia (CHN, LAOS, VIET), Japan (JPN) and Sri Lanka (SRIL).

Table 1
Geographic origin of the 95 *An. tessellatus* s.l. specimens examined in this study, with a summary of numbers of COI sequences and representative haplotypes in each cohort.

Country/State	Geographic code	No. of sequences	No. of haplotypes
China	CHN	15	14
Indonesia - Kalimantan	BO-KAL	20	15
Indonesia - Sulawesi	SUL	1	1
Japan	JPN	2	1
Laos	LAOS	1	1
Malaysia - Sarawak	BO-SAR	13	10
Malaysia - Sabah	BO-SAB	3	1
Philippines - Mindanao	PH-MIN	10	9
Philippines - Luzon	PH-LUZ	1	1
Singapore	SING	4	4
Sri Lanka	SRIL	3	3
Vietnam	VIET	22	22
Total		95	82

3.2. Species delimitation analysis

The ABGD analysis recovers two different partitions before grouping sequences as a whole (Supplemental Figure 1). The most conservative partition places sequences into two groups ("strict" ABGD partition). The final, most fragmented partition places sequences into four groups ("relaxed" ABGD partition).

The strict partition is recovered when the intraspecific (K2P) divergence prior is set at 1.36% (Fig. 2; Supplemental Figure 1; Supplemental Table 1). It partitions the Luzon (Philippines; PH-LUZ) and Sulawesi (Indonesia; SUL) sequences with 10 (of 20) sequences from Southeast Borneo (BO-KAL) and 11 (of 13) sequences from Northwest Borneo (BO-SAR) into the first group and places all remaining sequences into the second group. This first group is retained across all ABGD partitions, and across all analyses (ABGD, mPTP and RESL).

The relaxed partition occurs at intraspecific divergence priors of 0.50, 0.70 and 0.97% (Fig. 2; Supplemental Figure 1; Supplemental Table 1), and is therefore the majority partition for the ABGD analyses (3 out of 4 partitions). It retains the first group from the strict partition but splits the second group into three groups of Asian mainland + Sri Lanka (CHN, JPN, LAOS, SRIL, VIET), Borneo + Singapore (BO-KAL, BO-SAR, BO-SAB, SING) and Mindanao Island in the Philippines (PH-MIN).

Intra-specific diversity is very high across both partitions (up to 7.42% in the strict partition), but is minimized in the majority (relaxed) ABGD partition (Supplemental Table 1). However, even in this partition, the intra-specific diversity remains high for two of the four groups (3.65% and 4.22% maximum intra-specific K2P distance).

The mPTP partition separates sequences into 3 groups, placing in between the strict and relaxed ABGD partitions. It is similar to the relaxed ABGD partition but for merging the Borneo + Singapore (BO-KAL, BO-SAR, BO-SAB, SING) groups with that of Mindanao Island in the Philippines (PH-MIN).

The RESL clustering splits the sequence data into six putative species, labelled OTU-1 to OTU-6, and herein designated *An. tessellatus* A–F (Table 2; Fig. 2). The maximum intra-specific diversity is consistently low across all groups (maximum of 2.03%). The Borneo (OTU-3) and Singapore (OTU-4) groups are very closely associated, with minimum inter-specific variation < maximum intra-specific variation, negating the use of using simple pairwise distance thresholds for the discrimination of these species, and underscoring the potential problem with their use in even pairwise comparisons in general.

4. Discussion

Analyses of the barcode region of the *COI* mitochondrial gene using a range of species delimitation methods in this study supports the status of *An. tessellatus* as a species complex of up to six putative species. Identifying species complex structure in *An. tessellatus* and attributing epidemiologically important life history traits to particular species in the complex is fundamentally important for vector control and management strategies in the region.

Although all three analyses (ABGD, mPTP and RESL) yield different partitions, we consider the six-group partition from the RESL gives the most biologically meaningful intra-specific variation. Unlike partitions obtained from ABGD and mPTP analyses, the intra-specific variation from RESL analysis is comparable to the ~2% threshold for intra-specific

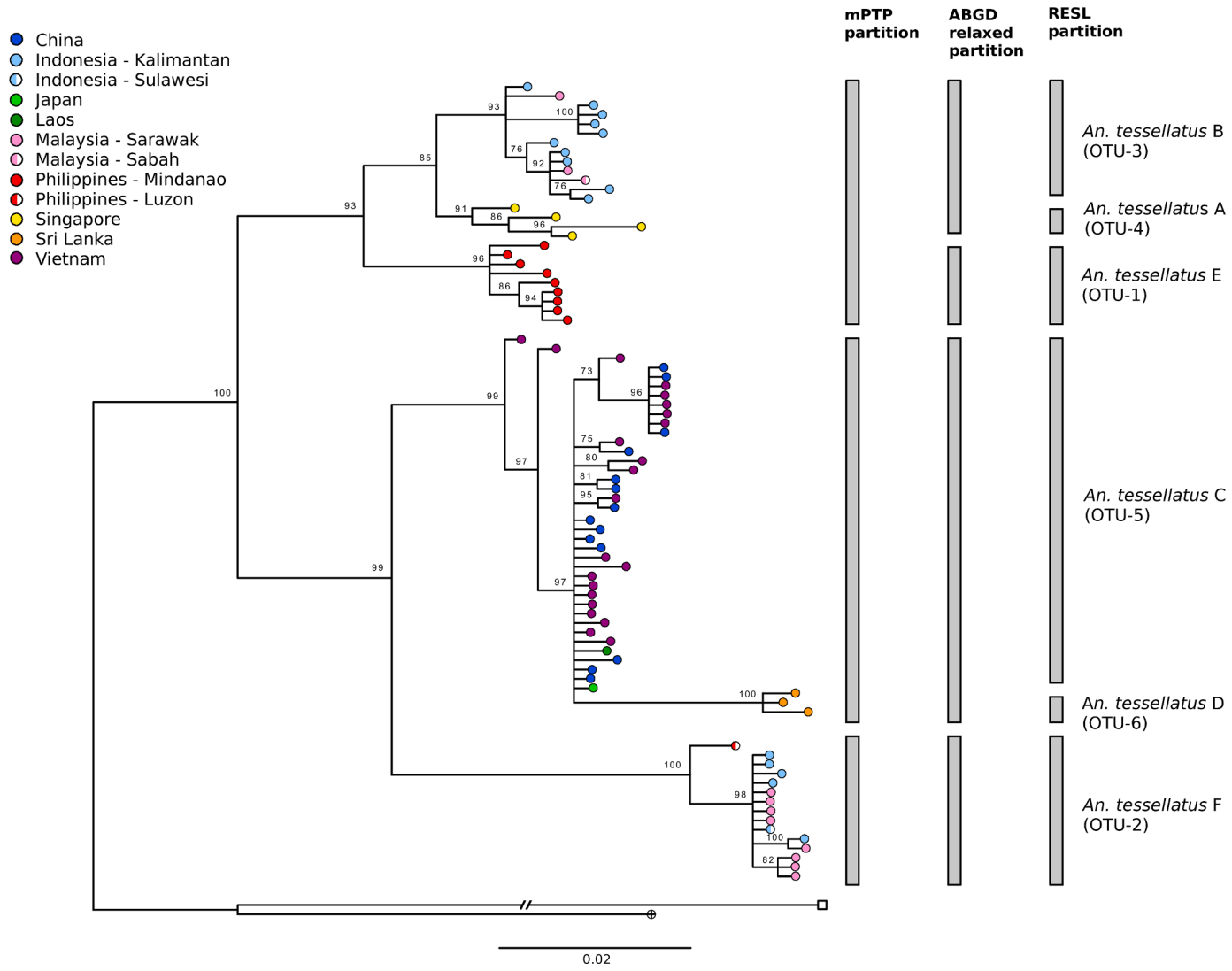

Fig. 2. Phylogenetic and species delimitation analysis of *An. tessellatus* s.l. sequences included in this study. Consensus tree (70% majority rule) from a Bayesian phylogenetic analysis using the COI gene (numbers at branches indicate Bayesian Posterior Probability, %). Haplotypes are colored according to their country/state of origin. *Chagasia bonneae* (□) and *An.* (*Nyssorhynchus*) *albitarsis* sensu stricto (⊕) were included as outgroup taxa. Multi-rate Poisson Tree Process (mPTP), Automatic Barcode Gap Discovery (ABGD) and Refined Single Linkage (RESL) delimitation is shown on the right.

Table 2
Species delimitation according to RESL analyses. Minimum inter-specific distance and mean and maximum intra-specific K2P distances are noted for each partitioned group.

Partition	Taxon	Putative_species	Origin_(n.=.number of.sequences)	Mean.intra-specific.distance	Maximum.intra-specific.distance	Minimum.inter-specific.distance	Nearest.neighbor
6 groups RESL	OTU1	*An. tessellatus* E	PH-MIN (n=10)	0.87	1.51	2.51	An. tessellatus B
	OTU2	*An. tessellatus* F	BO-KAL (n=10),BO-SAR (n=11), PH-LUZ (n=1), SUL (n=1)	0.23	1.38	3.87	*An. tessellatus* C
	OTU3	*An. tessellatus* B	BO-KAL (n=10), BO-SAR (n=2), BO-SAB (n=3)	0.95	1.86	1.34	*An. tessellatus* A
	OTU4	*An. tessellatus* A	SING (n=4)	1.42	2.03	1.34	*An. tessellatus* B
	OTU5	*An. tessellatus* C	CHN (n=15), JPN (n=2), LAOS (n=1), VIET (n=22)	0.67	1.72	1.96	*An. tessellatus* D
	OTU6	*An. tessellatus* D	SRIL (n=3)	0.49	0.73	1.96	*An. tessellatus* C

variation generally found in other studies of mosquito (Cywinska et al., 2006; Kumar et al., 2007; Wang et al., 2012) and insect COI diversity (Hebert et al., 2003; Huemer et al., 2014; Ratnasingham and Hebert, 2013). The six groups identified by RESL analysis, OTU-1 to OTU-6, are consequently assigned putative species status and denoted *An. tessellatus* A – F (Table 3; Fig. 3; see below). All but one of the six putative species identified form monophyletic clades – *An. tessellatus* C (CHN, JPN, LAOS, VIET) is paraphyletic with respect to *An. tessellatus* D (SRIL). Putative species fall into one of two basal clades. The first includes *An. tessellatus* A, *An. tessellatus* B and *An. tessellatus* E, and covers mainly insular Southeast Asia. The second clade comprises *An. tessellatus* C, *An. tessellatus* D, and *An. tessellatus* F, and is far more geographically diverse, covering South, Southeast and East Asia.

The most clearly and consistently resolved putative species in the analyses (most genetically distinct and delimited in all partitions) is *An. tessellatus* F (Fig. 2, Table 2, Supplemental table 1), found in Northwest and Southeast Borneo (Sarawak, Malaysia and Indonesian East Kalimantan, respectively), on the Indonesian island of Sulawesi and on the northern Philippine island of Luzon. This species is more closely related to geographically disparate putative species from mainland Southeast Asia, East Asia and Sri Lanka, than to geographically proximate putative species found in Borneo, the southern Philippine island of Mindanao, and Singapore. *Anopheles tessellatus* F is sympatric with *An. tessellatus* B, co-occurring in Northwest and Southeast Borneo (West Sarawak and East Kalimantan, respectively), which is likely to have implications for our current understanding of the ecology, and life history of the Tessellatus Complex on the island of Borneo.

Anopheles tessellatus is mostly considered zoophilic, being collected in high numbers in cattle-baited traps. However, in parts of Java, *An. tessellatus* populations display marked endophagic preferences (Stoops et al., 2009) and *An. tessellatus* was reportedly dominant in outdoor human landing catches in Sabah, Northeast Borneo, totaling 179 of 403 mosquitoes collected (Hawkes et al., 2017). The Sabah district is one of the sites where *An. tessellatus* B has been collected. Future studies of *An. tessellatus* on the island of Borneo will, therefore, need to consider the presence of these two highly distinctive clades present in Borneo (*An. tessellatus* B and *An. tessellatus* F), and further investigate potentially important differences in ecology, life history and vector competency.

The insect-specific flavivirus (ISF) Kampung Karu virus (KPKV)—a close relative of DENV and ZIKV (Guzman et al., 2018)—was recently isolated from a pool of *An. tessellatus* s.l. collected in Northwest Borneo (Kampung Puruh Karu, Sarawak) (Young et al., 2017). Although little is known about the epidemiological importance of ISFs, they may influence mosquito vector competence of human pathogens (Bolling et al., 2012; Hall-Mendelin et al., 2016), and their evolutionary potential for zoonoses is cause for speculation (Guzman et al., 2018). The location from where KPKV was isolated is proximate (~70km) to one of our sites in Northwest Borneo (neighboring Pandan and Siar Beaches), where we collected specimens of both *An. tessellatus* B and *An. tessellatus* F. We therefore cannot conclusively attribute the KPKV transmission to either species at this time.

The sites where *An. tessellatus* B and *An. tessellatus* F were collected are flanked by the type localities of *An. tessellatus tessellatus* to the west (Perak, Malaysia), *An. tessellatus kalawara* to the east (Sulawesi) and *An. tessellatus orientalis* to the east and south (Java and Sulawesi), as well as syn. *thorntonii* to the Northeast (Mindanao, Philippines) and syn. *deceptor* to the west (Sumatra). *Anopheles tessellatus* F was found to share the same island with the type localities of *An. tessellatus kalawara* and *An. tessellatus orientalis* on Sulawesi. However, deciphering clear relationships between *An. tessellatus* B and *An. tessellatus* F populations and the subspecies and synonyms in the complex will require a considerable amount of additional morphological and molecular analyses of museum and field collections from the region (Davidson et al., 2020).

Another highly resolved putative species is *An. tessellatus* E, identified from the large southern island of Mindanao in the Philippines, which lies close to the northeastern tip of Borneo. It is closely related and sister to a clade consisting of *An. tessellatus* A and *An. tessellatus* B, found in Singapore and in North and Southeast Borneo, respectively. It was not

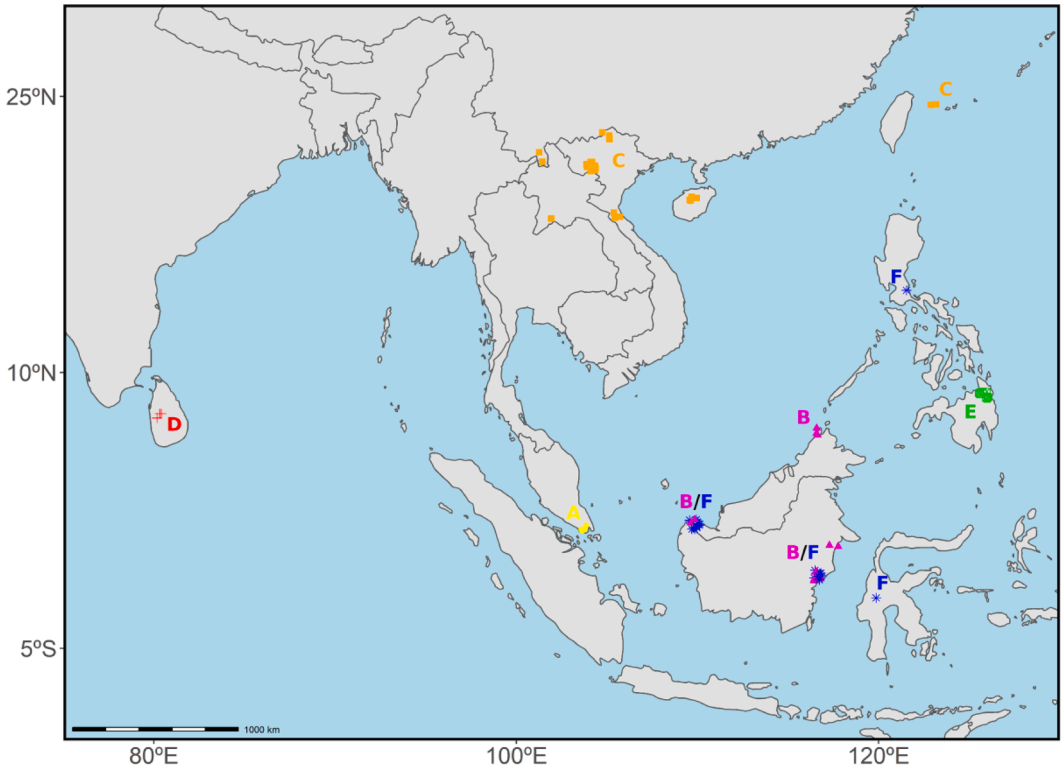

Fig. 3. Collection localities for the 95 *Anopheles tessellatus* s.l. COI sequences included in this study. Letters (A–F) are consistent with the lettering of putative species designation (Table 2). For illustrative purposes, locations have been jittered to reduce overplotting.

represented in samples examined from the northern Philippine island of Luzon (= *An. tessellatus* F), which is found more widely distributed on Borneo and on Sulawesi. The close proximity (~200km) of the collection site of *An. tessellatus* E (Bayugan, Agusan del Sur, Mindanao) to the type locality of *Anopheles thorntonii* (currently a synonym of *An. tessellatus*) in the province of Cotabato, Mindanao (Ludlow and Easton, 1904) suggests *An. tessellatus* E and syn. *thorntonii* may indeed be conspecific. Morphological studies are underway in our laboratory to determine if this putative species conforms to the original description of *thorntonii,* and the species should be elevated from its current synonymy with *An. tessellatus*. Whether *An. tessellatus* E and the *An. tessellatus* F (found on the northern Philippine island of Luzon) are sympatric in Mindanao, and across the Philippines, is unknown and assigning sibling species identity to the corresponding type specimen will require further collections across the archipelago. *Anopheles tessellatus* is considered a potential filarial parasite vector in the Philippines (Manguin et al., 2010), which has the heaviest lymphatic filariasis burden in the western Pacific (World Health Organization, 2010). The discovery of seemingly allopatric sibling species (*An. tessellatus* E and *An. tessellatus* F) in the Philippines, therefore, impacts our current understanding of *An. tessellatus* as a potential vector of filarial pathogens and our ability to incriminate it in pathogen transmission elsewhere in Luzon (Almarinez and Claveria, 2015; Chen et al., 2015; Walker et al., 1998) and many other Philippine islands such as Bongao (Salazar et al., 2015), Panay (Aure et al., 2016), Mindoro and Palawan (Baisas and Pagayon, 1956) where it is also found.

The *An. tessellatus* population found in Sri Lanka (= *An. tessellatus* D) is well documented as a competent vector of *P. falciparum* and *P. vivax* malaria (Amerasinghe et al., 1991; Gamage-Mendis et al., 1993) and a possible vector of Japanese Encephalitis virus (Banerjee et al., 1977) and filarial parasites (Abeyewickreme and Ismail, 1985) in the country. It is found near (~160km) the type locality of syn. *ceylonica* (= Trincomalee, Sri Lanka) (Newstead and Carter, 1910), which was later synonymized with *An. tessellatus* (Stanton, 1913). The current study therefore provides strong support for the description of this taxa as a species in its own right and morphological studies are underway in our laboratory to determine whether this is indeed a new taxa or requires the elevation of the prior name *ceylonica*. Given its geographic proximity and bionomic characteristics, it is possible that *An. tessellatus* D is the same as that found in the Maldives, which previously acted as the primary vector of human *Plasmodium* (Iyengar et al., 1953) and a formidable vector of filarial parasites (Iyengar, 1952) in the archipelago, but this remains to be confirmed.

Anopheles tessellatus C—identified from East and mainland Southeast Asia (China, Japan, Laos Vietnam)—occurs in regions where it is not implicated as a vector of human pathogens. Given the geographic proximity, one would be forgiven for assuming that samples from Taiwan (not included here) may likely be *An. tessellatus* C. However, three taxa that are currently in synonymy with *An. tessellatus* were previously described from Taiwan. These include syn. *formosae* Hatori 1901, syn. *kinoshitai* Koidzumi 1917, and syn. *taiwanensis* Koidzumi 1917, suggesting considerable morphological variation between these, and the possibility that several species are, or have been, present on the island. Further molecular studies that include populations from the type localities in modern day Pingtung City (syn. *taiwanensis* Koidzumi 1917; locality: Banshoryo, Ako) in the south, and the district of Tamsui (syn. *formosae* Hatori 1901; locality: Tansui) and the province of Tapei (syn. *kinoshitai* Koidzumi 1917; locality: Ryukokosho) in the north are necessary before the taxonomic status of the Taiwanese *An. tessellatus* population found naturally infected with Japanese Encephalitis virus (Su et al., 2014) can be correctly assigned.

Although the scope of our study was limited to COI data, which was routinely collected for species barcoding and surveillance initiatives, the examination of several publicly available *An. tessellatus* ITS2 sequences (AB731657, EU650425, MN203103, MT623072-MT623074) from locations close to some our collection sites provided some additional support for extant inter-specific diversity and our putative species designations. Chinese and Vietnamese sequences were almost identical (single base difference), but p-distances showed these were 1.7% and 10.5% different from invariable *An. tessellatus* sequences collected in Sulawesi, Indonesia and in Sabah, Malaysia, respectively. Difficulties with ITS2 sequence alignment due to high levels of polymorphism even among closely related species (Bourke et al., 2011) coupled with the potential presence of incomplete concerted evolution and resulting intragenomic variation (Li and Wilkerson, 2007; Motoki et al., 2011) can confound phylogenetic reconstruction using ITS2. However, the available *An. tessellatus* ITS2 data indicates the possible phylogenetic utility of this locus in further systematic studies of the Tessellatus Complex.

The geographical distribution of putative species in the *An. tessellatus* complex appears to conform, to some degree, to the biogeographic hotspots defined by Myers et al. (2000), which are considered important for defining the *Anopheles* species turnover in Southeast Asia (Morgan et al., 2013). *Anopheles tessellatus* C and *An. tessellatus* D are identified from the Indo-Burma and Western Ghats/Sri Lanka hotspots, respectively (although see the inclusion of Yonagunijima, Japan specimens in the former). *Anopheles tessellatus* A and *An. tessellatus* B are both currently limited to the Sundaland hotspot, while *An. tessellatus* E is identified from the Philippines hotspot. Interestingly, *An. tessellatus* F overlaps all three biogeographic regions found in insular Southeast Asia, traversing the Sundaland, Wallacea and the Philippine hotspots. Some of this distribution is perhaps indicative of the Philippine island of Palawan, which runs close to the coast of Northeast Borneo, serving as a historical conduit or colonization route from Sundaland into the Philippines, or vice versa.

Unfortunately, due to the limited number of collection sites in this preliminary study, we cannot assume that the geographic partitioning of putative species in our study are true representations of species distribution. Three of the six putative species (*An. tessellatus* A, *An. tessellatus* D and *An. tessellatus* E) have only been detected at a single locality and, although *An. tessellatus* F has been collected from multiple disparate localities in Borneo, it has only been collected from a single locality in the Philippines (Luzon) and on the Indonesian island of Sulawesi. In the case of the Philippines, although sample localities were located on two of the largest islands, the archipelago is made up of more that 7,000 islands scattered across nearly 2 million square kilometers (Central Intelligence Agency, 2020). We therefore cannot claim our findings represent species turnover between islands in northern and southern Philippines.

Given the limitations of sampling in the current study, it is not possible to test for the importance of the major biogeographic boundaries, which are believed to be important in shaping species diversity in the region. A more comprehensive study of the Tessellatus Complex (formally assigned herein) across its geographic range would provide clearer insights into biogeographic patterns and historical routes of dispersal and colonization. We propose additional sampling in islands that are currently only represented by a single locality (Luzon, Mindanao, Sulawesi and Sri Lanka), and in Sumatra, Java and the Malaysian peninsula to better represent diversity in Sundaland.

In order to fully characterize all component members in the Tessellatus Complex, it is essential first to ascertain the genetic identity of *An. tessellatus* s.s. by obtaining DNA sequences from topotypic exemplars at, or close to, the type locality of in Taipang, Perak, northern Malaysia. Only when the identity of the nominotypical taxon is ascertained, can we move forward with the formal elevation and/or naming of the six sibling taxa described herein. Studies are underway in our laboratory to correlate morphotypes with the molecular forms described herein, and will be elaborated in a follow-on manuscript. Although our findings are derived from a single locus, our study nonetheless provides a baseline for genetic diversity in *An. tessellatus* across a considerable portion of its geographical range and support for the existence of multiple members of the Tessellatus Complex for the first time, emphasizing the utility of DNA barcoding for the recovery of hidden biodiversity in mosquitoes.

These findings form the starting point of a comprehensive systematic investigation of the Tessellatus Complex using an integrated morphological study combined with a multi-locus or genomics-based approach that will provide deeper insight into the species diversity and evolutionary history of this complex. Highly significant processes such as incomplete lineage sorting and introgression compound efforts to reconstruct species phylogenies (Degnan and Rosenberg, 2009; Funk and Omland, 2003), and analyses that incorporate genome-wide data and take account of such processes can provide, not only more robust phylogeny and species delimitation, but also an understanding of the historical biogeographical processes that shaped diversity in this species complex (Andrews et al., 2016; Cruaud et al., 2014; Eaton and Ree, 2013; Wagner et al., 2013).

CRediT authorship contribution statement

Brian P. Bourke: Conceptualization, Data curation, Formal analysis, Visualization, Writing - review & editing. **Richard C. Wilkerson:** Conceptualization, Writing - review & editing. **Yvonne-Marie Linton:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

All authors hereby declare no conflict of interest.

Acknowledgment

We are thankful to Elizabeth Torres, Chang Moh Seng, Asmad Matusop, Nguyen Duc Manh, Marc Coosemans, Wim Van Bortel, Cathy Walton, Ralph Harbach, Tom Anthony, Magdalena Zarowiecki, Fredy Ruiz-Lopez, Renita Danabalan, and Annette Lee who all participated in and/ or facilitated field studies and projects where these non-target samples were collected. COI sequences were generated through the sequencing facility led by Julia Llewellyn Hughes at the Natural History Museum (UK). The study was funded by the Armed Forces Health Surveillance Division (AFHSD) - Global Emerging Infections Surveillance (GEIS) Branch research project award P0140_20_WR_05 (to YML); BPB is a National Research Council Senior Research Associate at the Walter Reed Army Institute of Research (WRAIR). The analyses were performed in part under a Memorandum of Understanding between the Walter Reed Army Institute of Research (WRAIR) and the Smithsonian Institution, with institutional support provided by both organizations. The material published reflects the views of the authors and should not be misconstrued to represent those of the U.S. Department of the Army, and the U.S. Department of Defense. The funders had no role in study design, data collection, and analysis, decision to publish, or preparation of the manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2020.105799.

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