REVIEW

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A systematic review: is *Anopheles vagus* a species complex?



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Abstract

Background Anopheles vagus (subgenus Cellia) has been identified as a vector for malaria, filariasis, and Japanese encephalitis in Asia. Sporozoites of *Plasmodium falciparum* and *Plasmodium vivax* have been found in this zoophilic mosquito in Asia and Indonesia. This study systematically reviews publications regarding *An. vagus* species, variation, bio-ecology, and malaria transmission in various localities in Asia, especially Indonesia, to determine whether the current data support *An. vagus* as a species complex.

Methods The databases Pubmed, Scopus, Europe PMC, and Proquest were searched to identify information regarding the morphology, karyotypes, polytene chromosome, cross-mating, ecology, and molecular identification of *An. vagus* was then evaluated to determine whether there were possible species complexes.

Results Of the 1326 articles identified, 15 studies were considered for synthesis. The *Anopheles* spp. samples for this study came from Asia. Eleven studies used morphology to identify *An. vagus*, with singular studies using each of karyotype identification, chromosomal polytene identification, and cross-breeding experiments. Ten studies used molecular techniques to identify *Anopheles* spp., including *An. vagus*. Most studies discovered morphological variations of *An. vagus* either in the same or different areas and ecological settings. In this review, the members of *An. vagus* sensu lato grouped based on morphology (*An. vagus, An. vagus vagus, An. vagus limosus*, and *An. limosus*), karyotyping (form A and B), and molecular (*An. vagus* genotype A and B, *An. vagus* AN4 and AN5). Genetic analysis revealed a high conservation of the ITS2 fragment among members except for the *An. vagus* genotype B, which was, in fact, *Anopheles sundaicus*. This review also identified that *An. vagus limosus* and *An. vagus* were nearly identical to the ITS2 sequence.

Conclusion Literature review studies revealed that *An. vagus* is conspecific despite the distinct morphological characteristic of *An. vagus* and *An. limosus*. Further information using another barcoding tool, such as mitochondrial COI and ND6 and experimental cross-mating between the *An. vagus* and *An. limosus* may provide additional evidence for the status of *An. vagus* as a species complex.

Keywords Anopheles vagus, Anopheles vagus limosus, Anopheles limosus, Species complex, Sibling species, Phylogeny

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Background

Malaria is an infectious disease caused by Plasmodium spp. transmitted by female Anopheles mosquitoes. Southeast Asia has the second highest malaria incidence after Africa, with an estimated eight million cases and 11,600 malaria deaths [1]. Globally, a total of 465 Anopheles malaria vectors have been identified morphologically, with 70 species from the four subgenera Anopheles, Cellia, Kerteszia, and Nyssorhynchus transmitting the malaria parasite to humans [2]. Anopheles species are often found in morphologically identical sibling species complexes, with approximately thirty species complexes identified in various parts of the world [3-6]. Each complex has a different number of sibling species, and 145 species have been identified [2]. The accuracy of morphological identification depends on the known ability to differentiate species and those held within species complexes cannot usually be differentiated morphologically. Cross-mating tests, mitotic and meiotic karyotypes, and molecular procedures are applied to identify sibling species within complexes [3–5]. Sibling species held within complexes often express different behaviours and bionomics, therefore, it is crucial to utilize molecular techniques to identify the mosquito specimens to species level, when examining the mosquito geography, ecology, and biology [3, 6].

More than eighty *Anopheles* species were identified in Indonesia, of which twenty-six are malaria vectors. So far only a few species have been studied as *Anopheles* species complex in Indonesia, including *Anopheles sundaicus*, *Anopheles maculatus*, *Anopheles barbirostris* and *Anopheles punctulatus* [7–13]. This does not rule out the possibility of other complexes that have not been discovered yet.

Anopheles vagus (sub-genus Cellia) was discovered in 1902 by Doenitz in Indonesia [14], and its subspecies, An. vagus limosus was initially reported in the Philippines [14, 15]. In Indonesia, An. vagus is a vector of malaria, filariasis, and Japanese encephalitis [16–18]. Except for Papua, practically all Indonesian Islands have An. vagus populations [19], which typically feed on cattle and other animals. These primary topographic zones comprise a diverse habitat, including brackish water, coastal plains, inland, hills, and mountains [20]. Anopheles vagus larvae are generally found in locations with calm or light water flow, such as puddles, on the beach, springs, the edges of rice fields, muddy ponds, animal tracks, and artificial containers such as old tires, drums, and on boats [20–22].

Anopheles vagus is predominantly a zoophilic, exophilic, and exophagic vector found in Asia [19]. However, in some previous studies, *An. vagus* was also reported to be slightly more anthropophilic, i.e. feeding on human blood and/or animal blood [23, 24]. This

opportunistic behaviour can make these mosquitoes capable of transmitting *Plasmodium*. Therefore, these mosquitoes are now regarded as secondary malaria vectors, after the detection of *Plasmodium* spp. both by biological assays and molecular examination, which has been carried out in several regions in India, Bangladesh, Thailand, China, and especially Indonesia [19, 25–31].

This review evaluated the current evidence to determine whether *An. vagus* was a cryptic or sibling species, as well as the behavioural and genetic variation of *An. vagus* in Asia.

Methods

Literature search

This review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) 2020 guidelines [32]. The databases Pubmed, Scopus, Europe PMC, and Proquest were searched using the following keywords: "(Anopheles) AND (Anopheles vagus)"; "((species complex) OR (sibling species)) AND (Anopheles vagus)". The search was conducted from February to November 2022.

Eligibility criteria and study selection

English and Indonesian language full articles concerning *An. vagus*, various systematic examinations of species complex based on karyotypic identification, cross-mating experiments, morphometric and morphological investigations of palps and wings, and molecular investigations for identifying species and phylogenetic analysis were evaluated. Three authors (DL, DS, CA) independently extracted data regarding authorship, years, country, study populations, and key findings from each study.

Results

In total, 145 articles were identified in Pubmed, 192 in Scopus, 642 in Europe PMC, and 347 in Proquest, with a further six relevant studies in Google Scholar (Fig. 1). Most studies were about Anopheles spp., focusing on bionomy, survey mosquitoes and their role as a vector, incrimination study, abundance and diversity, and molecular analysis. Only a few articles focused on An. vagus and the systematic study of the possibility of a species complex. Of the fifteen studies reviewed [33-47](Table 1), seven studies examined a sample of An. vagus from the Indonesian archipelago (Central Java, East Java, and West Sulawesi Indonesia as well as Dili East Timor), four studies involved samples from India, two from Thailand, one study from the population in 18 areas of mainland Asia and Southeast Asia, and one study from the border regions of Laos and Cambodia (Fig. 2). Of the fifteen articles on An. vagus, all have used different species name in the articles and in the genebank, such as An.





Fig. 1 PRISMA flow diagram of literature searching

vagus. An. limosus, An. vagus vagus and An. vagus limosus [36, 37, 42].

In this review, the members of *An. vagus* sensu lato (s.l.) grouped based on the morphology (*An. vagus, An. vagus vagus, An. vagus limosus,* and *An. limosus*), karyotyping (form A and B), and molecular (*An. vagus* genotype A and B) and *An. vagus* AN4 and AN5.

Current grouping of Anopheles vagus

Female *An. vagus* was initially discovered by Doenitz (1902) in Sumatera, meanwhile the male *An. vagus* was discovered in Java and other regions in Indonesia [14]. Based on morphological features, *An. vagus* can be divided into three subspecies: *An. vagus vagus*, *An. vagus limosus* and *An. vagus albino* [48]. *Anopheles vagus limosus* was found in Luzon Island, Philipines, and *An. vagus vagus* was found in Lake Lanao, located in Lanao Plateau, Philippines [49]. The status of *An. vagus limosus* was later promoted as a new species by Ramalingan et al. [50], who collected the two subspecies at the sympatric speciation in Sabah, Malaysia. This separation was reinforced by developing the cladistic classification of the genus *Anopheles*, which placed *An. vagus* and *An. limosus* into different species but still siblings in Pyretophorus series based on morphological and morphometric characters [51–53].

Morphology-based grouping of Anopheles vagus

One of the distinctive characteristics of the genus *Anopheles* is its morphological variety. *Anopheles vagus* differs from other species in the Pyretophorus series, such *An. indefinitus* and *An. subpictus* from its pale apical band on its proboscis. It also differs from *An. limosus* from it lacks spots on its femur and tibia. *Anopheles vagus* has three or more palpi with pale or dark bands, a pale band at the proboscis's tip, and a pale band between the two dark bands on the humeral wing [54]. The species of *An. vagus* and *An. limosus* are distinguished by the proboscis and prehumeral region variations, which may be a

Table 1 Summary and key findings included	irticles			
Techniques for identification mosquito siblings	Authors/years	Population size/sample site	Key findings	References
Metaphase karyotypes	Baimai et al. 1996 ^a	Anopheles subgenus Cellia from Thailand, Indone- sia, Filipina, and Bangladesh, for An. vagus sample from Thailand (Songkhla and Nakhon Nayok province)	Two forms of isoline F1 <i>An. vagus</i> progeny: form A from southern Thailand Chiangmai and Song- khla; form B from Nakhon Nayok Province, Thailand	[33]
Intraspecific hybridization	Choocote et al. 2002 ^a	<i>An. vagus</i> from San Sai and San Kamphaeng Dis- tricts, Chiang Mai Province, northern Thailand	All crosses yielded viable progeny, with no evidence of genetic incompatibility between An. vagus forms A and B from a different area (allopatric). The eggs of An. vagus forms A and B were morphometrically and morphologically identical	[34]
Polytene chromosome and molecular	Paul & Banerjee, 2016 ^b	<i>An.vagus</i> from west Bengal, India	<i>An. vagus</i> from a different area (allopatric) in west Bengal has a different arm structure of the poly- tene chromosome, with the presence of tetram- ers, pentamers, polymers, and the absence of repeats in the ITS2 sequence	[40]
Morphology	Jagdish Kaur, 2015 ^b	<i>An. vagus</i> and <i>An. fluviatils f</i> rom Punjab, Haryana, Uttarakhand, and Himachal Pradesh, India	Thirteen morphological variations were observed in the ornamentation of the wings and palpi (7 variations in the wings and palpi of <i>An. vagus</i> and 6 wing variations in <i>An. fluviatilis</i> (both from allopatric and sympatric area)	[41]
Morphology	Wahyuni et al. 2018 ^b	<i>An. vagus vagus</i> and <i>An. vagus limosus</i> from Banyu- wangi East Java, Indonesia	<i>An. vagus</i> found in Bangsring Village does not have a combination variation in the proboscis and prehumeral wings. <i>An. vagus vagus</i> is charac- terized by unspotted legs, a proboscis with pale bands, and prehumeral wings with pale bands between two dark bands, whereas <i>An. vagus</i> <i>limosus</i> has characteristic unspotted legs, a dark overall proboscis, and prehumeral wings with dark bands; there is no mixed combination of these characteristics between the two species	[42]
Morphology and ecology	Siti Alfiah & Mujiyono, 2014 ^b	<i>An.vagus</i> from Semarang, Central Java Indonesia	The variations were in the size and the number of hair branches and filaments. This variation in the intra and interpopulation An. vagus in fresh and brackish water was caused by the difference in geographical location (allopatric speciation)	[43]
Morphology, ecology, and molecular	Cooper et al. 2010 ^a	Anopheles spp from Dili East Timor	Analysis of ITS2 and Cyt b revealed the <i>An. vagus</i> genotype A (mainly found inland and genetically similar to <i>An. subpictus</i>) and <i>An. vagus</i> genotype B (dominant in HLC and + sporozoite, mainly found on the coast and brackish water genetically similar to <i>An. sundaicus</i>)	[44]

Techniques for identification mosquito siblings	Authors/years	Population size/sample site	Key findings	References
Morphology and molecular	Zarowiecki et al. 2011 ^a	<i>An. wagus</i> from 18 populations in 10 countries in mainland Asia and Southeast Asia	Analysis of COI (Cytochrome Oxidase I) and ITS2 (Internal Transcribed Spacer 2) revealed that <i>An. vagus</i> appears to reflect a highly diverse, monospecific, widespread taxon distributed in Sri Lanka and India throughout mainland and Southeast Asia, except Java and East Timor (<i>An. vagus</i> FJ654649), which forms a distinct genetic lineage with the sample from other regions. There is some degree of east-west genetic differentiation in <i>An. vagus</i> along the Thai-Myanmar border due to historical allopatric fragmentation	[4.5]
	Zomuanpuii et al. 2013 ^a	<i>Anopheles</i> subgenus Cellia (10 species) from 5 districts in Mizoram, India	Analysis of ITS2: An. vagus had the longest ITS2 regions but possesses low repeats and polynucleotide microsatellites (no dimer repeats found in An. vagus)	[46]
	Paul et al. 2015 ^b	<i>An. vagus</i> and <i>An. subpictus</i> from Morga India	3 different palp types from two seasons (summer and monsoon) in 7 samples; <i>An. vagus</i> is geneti- cally similar to <i>An. subpictus</i>	[47]
	Davidson et al. 2020 ^a	Anopheles spp from Karama, West Sulawesi Indone- sia	Analysis of COI and ITS2 sequence: 2 distinct groups identified as <i>An. vagus</i> (AN4 and AN5) from this sympatric area, with <i>An. vagus</i> (AN4) more closely related to <i>An. sundaicus</i> (AN17) and <i>An. vagus</i> (AN5) more closely related to <i>An. subpictus</i>	[3.5]
	Senjarini et al. 2021 ^b	<i>An. vagus vagus</i> and <i>An. vagus limosus</i> from Banyu- wangi, East Java Indonesia	Analysis of ITS2 sequence: <i>An. vagus vagus</i> and <i>An. vagus limosus</i> closely related to <i>An. vagus</i> FJ654649 and were in the same clade	[36]
	Senjarini et al. 2021 ^a	<i>Anopheles spp</i> from Banyuwangi, East Java Indo- nesia	Analysis of Sma-ITS 2: An. vagus vagus, An.vagus limosus, and An. indefinitus formed a single clade with no clear boundaries between An. vagus vagus and An. vagus limosus	[37]
	Zhang et al. 2022 ^a	Anopheles spp. from the Cambodia-Laos border	An. vagus was the dominant species but only 12 An.vagus were randomly investigated by molecu- lar analysis of ITS 2 and the COII sequence: all are not genetically distinct An. vagus-like species	[38]
	Hasanah et al. 2022 ^a	An. <i>vagus,</i> An. <i>subpictus,</i> An. <i>sundaicus,</i> and An. <i>aconitus</i> from basring village Banyuwangi, East Java, Indonesia	Analysis of ITS2 sequence: <i>An. vagus</i> and <i>An. aconi-</i> <i>tus</i> were monophyletic and <i>An. subpictus</i> and <i>An.</i> <i>sundaicus</i> were polyphyletic	[39]
^a Studies from the Pubmed, Scopus, Europe PMC and Pro ^b Study from Google scholar	Quest databases			





Fig. 2 Geographic Distribution Anopheles spp. and Anopheles vagus examination. Map of Country in Mainland Asia and South East Asia was sourced from: https://gadm.org/maps.html

combination of traits from two species specimens from Java, Indonesia [45]

The current study from India found that the An. vagus palp variation is discernible from the uneven and black tip palp [47]. The size and number of the collected branches, hairs, and filaments may vary depending on whether the collected An. vagus specimens were from freshwater or brackish water or due to the season or geography [41, 43, 47]. However, in Bangsring, East Java, the sample population taken from an area near a brackish water pond showed that the two subspecies of An. vagus do not have a combination variation in the proboscis and prehumeral wings. Anopheles vagus vagus is characterized by unspotted legs, a proboscis with pale bands, and prehumeral wings with pale bands between two dark bands, while An. vagus limosus has characteristic unspotted legs, a dark overall proboscis, and prehumeral wings with dark bands [42].

Karyotype grouping of Anopheles vagus

Several mosquitoes of the subgenus *Cellia*, including a few *An. vagus* mosquitoes from Thailand were used in the initial studies on metaphase analysis of karyotypes [33]. Two distinct *An. vagus* karyotypes, form A and form B, were identified with a different length of the chromosomal arm. However, it has yet to be determined if the two metaphase karyotypes represent intra- or interspecies variations [33].

Experimental intraspecies cross-mating was conducted to identify the post-mating barriers between isolines of *An. vagus* types A and B from the districts of San Sai and San Kamphaeng, Chiang Mai Province, Northern Thailand. The results revealed that the *An. vagus* eggs are homogeneous with both forms, proving that the *An. vagus* species has two cytological forms that are polymorphic races [34]. In addition to karyotyping, there is only one study that analyses polytene chromosomes in *An. vagus*. Therefore, the result must be different from other studies. In that study, it was revealed that distinct polytene chromosomal arms variation in banding and puffing patterns with tetramers, pentamers, and polymers present but no tandem repeats in the ITS2 sequences [40].

Molecular grouping of Anopheles vagus

The advent of molecular technologies in the early 1990s has provided new insights into mosquito taxonomy and further strengthened the taxonomic classification mainly based on morphological features. Molecular barcoding using either the internal transcribed spacer 2 (ITS2) fragment of the nuclear ribosomal gene or the mitochondrial cytochrome oxidase I (COI), and NADH dehydrogenase subunit 6 (ND6) gene offered complementary evidence to the current taxonomic classification and identification of a cryptic species [55–58].

Anopheles vagus appears dominant in mainland and Southeast Asia populations comprising a molecularly highly diverse yet monospecific, widely dispersed taxon. Interestingly, specimens from Java and East Timor in the Indonesia Archipelago region had a unique genetic lineage to other An. vagus in Asia [45]. Anopheles vagus had a highly conserved ITS2 region containing over 500 bp and flanked by 5.8S rDNA in the upstream and large rDNA in the downstream (Fig. 3). A recent study by An. vagus ITS2 revealed no genetic variations among the An. vagus populations from Bangsring, East Java, Indonesia [39]. Likewise, there were no genetically distinct An. vaguslike species in twelve specimens from the border between Laos and Cambodia [38]. In contrast, studies conducted in East Timor [44] reported two groups of An. vagus based on ITS2 sequence; Genotype A perfect match with sequence of An. vagus from East Timor and East Java (GenBank accession number FJ654649), whereas Genotype B was morphologically identical to An. vagus, but the ITS2 sequence was nearly identical to An. sundaicus (Fig. 4). The An. vagus genotype B was reportedly more anthropophilic than the An. vagus genotype A as it was more frequently collected through the HLC technique.

Interestingly, two groups of sympatrically distributed *An. vagus* was also reported in the remote inland village of Karama, West Sulawesi, Indonesia; one was closely related to *An. sundaicus*-like, namely AN4, while the other was related to *An. subpictus* like (AN5) [35]. However, no further information as to whether the two *An. vagus* subgroup was reproductively isolated [47]. Both these studies used the genetic marker internal transcribed spacer 2 (ITS2). These studies also used COI sequences to identify their phylogenetic relationship.

Anopheles vagus vagus and An. vagus limosus from Bangsring, East Java, Indonesia are morphologically different, but their phylogenetic relationship was very close with ITS2 sequence similarity of more than 99%. Both *An. vagus vagus* and *An. vagus limosus* ITS2 sequence resembles with the previously published *An. vagus* ITS2 sequence (GenBank accession number FJ654649) [20, 23].

Discussion

The biological and ecological notion of allopatric speciation which enables the *Anopheles* species to be separated in an ecological niche, involves ecosystem patterns, geographic barriers, and varied terrain that result in physical separation and reproductive isolation [3, 6, 59, 60]. Initially, it was believed that it was unusual for gene flow to remain uninhabited; however, it is now widely acknowledged that sympatric speciation has occurred possibly involving interbreeding speciation and assortative mating by habitat or secondary gene flow [61–63].

Anopheles vagus currently possesses several subspecies based on morphological characteristics and karyotype. Karyotype forms A and B are distributed in allopatric and thus possess geographical barriers to mating but have been shown through laboratory crossmating to produce viable offspring [35] thus, the finding they concluded that *An. vagus* form A and form B from allopatric speciation are likely sibling species rather than different species entities [34]

Morphological characteristics divide *An. vagus* into two subspecies; *An. vagus vagus* and *An. vagus limosus* [14, 15]. The latter subspecies was later promoted into a new species, *An. limosus* (based on Harbach classification for *Anopheles*) [2, 51–53]. *Anopheles vagus* and *An. limosus* were distributed sympatricly in several parts of Southeast Asia [42, 45, 50].

Analysis of ITS2 sequences of all members of *An. vagus* s.l. revealed that *An. vagus, An. vagus vagus, An. vagus limosus* and *An. limosus* share almost identical ITS2 sequences except for *An. vagus* genotype B is, in fact, a member of *An. sundaicus* [23]. Therefore, despite the distinct morphological variation, all members of *An. vagus* are possibly conspecific. Regarding *An. limosus* that has been given new species status, further species confirmation using the other barcoding marker such as COI and ND6 may support this new status. The other issue that is also important is whether the *An. vagus* and *An. limosus* naturally mate with each other. So far, despite their close relationship, there is no evidence that they could mate naturally or in a laboratory setting that produces viable offspring.

Conclusion

Literature review studies revealed that *An. vagus* is possibly conspecific despite distinct morphological characteristics of *An. vagus* and *An. limosus.* Further information using mitochondrial COI and ND6

	and a Tana a	10	20	. 1	30	40		50		60		70		30		90	i	100
An. ragus (FJ654649)	ACCGATGCA	CACATCCT	TGAGTG	CTACT	AGGTAC	TGAGATT	TAACT	ATGACT	TGACT	CAGA	GSCC	SCCAC	TAAA	GGGCT	GACGG	GCCAT	CGTCG	TC
An. ragus (OM974188																		
An. ragus type B(1)					 	TC	TC.	AT	A		G		c			T		
An ragus type B(2)						. TC	. TC	A . T .	A		<mark>G</mark> .		<mark>C</mark>			T		• •
An. ragus AN4 An. ragus AN5																		
An. v. vagus (I)																		
An. r. ragus (2)				• • • • •														
An limosus																		
																_		
		110	120		130	140		130		160		170		150		190		200
An. ragus (F]654649)	CGGCGTGCG	ACTGTGCA	GCATGG	GTGCT	CGGGTC	TCGGCGT	GGACO	CTTGGG	CGCTG	AAGT	GACAG	СТОС	TTTG	GCGG	CACCT	GCGCG	GTGCT	CT
An. vagus (OM974188	2											· · · ·				•••••		
An. ragus type B(1)	Τ										. т.	ст		A		тт		
An. ragus type B(2)	Τ										T	СТ.		Α		ΤΤ		
An. ragus AN4 An. ragus AN5													1111	1				
An. r. ragus (1)																		* *
An. r. ragus (2)			*****								· • · • • • •	•••••						
An. limosus																		
	-										-	_				_		
	1	10	220		230	240		250		260		270		280		290		300
An. ragus (FJ654649)	C A GT GT	TGACGTAT	GGTGAG	GTAGT	GTCAAG	TCGCACO	GTTCC	ACAACA	AGC	STACC	TCGAG	STTTC	GTGC	AATCG	GATGC	CTACT	CCATG	GG
An. ragus (OM974188																		
An. ragus type A An. ragus true B(1)	СТА	c				c	G		CA	· · · · ·								
An. ragus type B(2)	. CTA	с				с	G		СА	Т								
An. ragus AN4		• • • • • • • •					•••••									• • • • •		• •
An. r. raeus (I)																		
An. v. vagus (2)				W														
An. v. limosus An. limosus																		•••
100 0000						-												
	1	120	3.20	1	330	340		350		360	1	370		330		310		+00
Ап. тадия (F]654649)	CGGTGCCGG	CGTGCATT	120 CAACAC		JIO	340	CCAAC	CGGATG	cc	340	GTGA	370 GC	CGGT	жо СССССС	CGCAG		CACTG	400 A A
Ап. тадия (FJ654649) Ап. тадия (ОМ974188	cGGTGCCGG	CGTGCATT	320 CAACAC/		330 TCGACC	340 TCCCGTA	CCAAC	CGGATG	c c	340	GTGA	370 GC	CGGT	330 GCCGG	CGCAG		CACTG	400 A A
An. ragus (FJ654649) An. ragus (OM974188 An. ragus type A An. ragus type B (1)	cGGTGCCGG		320 CAACAC	GACO	TCGACC 	J40 TCCCGT/	CCAAC	350 C G G A T G	C C	340 GCCG	GTGA	370 GC 3TT	GGT		CGCAG	310 A C G G G J	G. GC	400 A A G C
An. ragus (FJ654649) An. ragus (OM974188 An. ragus type A An. ragus type B (1) An. ragus type B (2)	caataccaa		320 CAACAC	AC GACC	330 TCGACC GCGT GCGT	340 TCCCGTA T.	ССААС	350 C G G A T G	сс 	340 GCCG	GTGA	370 GC 3TT 3TT	CGGT	330 GCCGG . T	CGCAG	310 A C G G G G 	G. GC	400 A A G C G C
An. ragus (F[654649) An. ragus (OM974188 An. ragus type A An. ragus type B (1) An. ragus type B (2) An. ragus AN4 An. ragus AN4	CGGTGCCGG		320 CAACACA	GACC	330 TCGACC GCGT GCGT	340 5T C C G T A T T		350 C G G A T G	C C 	360 I GCCG	GTGA 	370 GC GTT GTT	CGGT	GCCGG	CGCAG	300 A C G G G G A A	G. GC G. GC	400 A A G C G C
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Fig. 3 Sequences Alignment rDNA ITS2 fragment sample *An. vagus* members from Indonesia and East Timor. *An. vagus* (GenBank accession number FJ654649) is the reference sequence from all studies from Indonesia and East Timor. *An. vagus* (OM974188) is newest sequence sample from East Java Indonesia. GenBank accession number GQ500122 for *An. vagus* genotype A, GQ480824 and GQ480823 for *An. vagus* Genotype B, MT740902 and MT740903 for *An. vagus* AN4 dan AN5, MW314227 and OL437110 for *An. vagus vagus* (1) and (2), MW319822 for *An. vagus limosus*. OL437109 for *An. limosus*

barcoding and experimental cross-mating between the *An. vagus* and *An. limosus* may provide additional evidence for the status of *An. vagus* as a species complex.

Abbreviations

- ITS2 Internal transcribed spacer 2
- COI Cytochrome oxidase I
- HLC Human landing catch
- ND6 NADH dehydrogenase subunit 6

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Author contributions

The authors confirm their contribution to the paper as follows: study conception and design DL and DS; data collection: DL; analysis and interpretation of results: DL, DS and CA; draft manuscript preparation: DL, DS, CA, AG, LV, LS, IS. All authors reviewed the results and approved the final version of the manuscript. All authors were involved in the revision of the draft manuscript and have agreed to the final content.



Fig. 4 Sequence alignment rDNA ITS2 fragment An. sundaicus (AY768540) and An. vagus genotype B (GQ480823 and GQ480824)

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Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication

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Competing interests

The authors declare that they have no conflict of interest.

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