

MOLECULAR VARIATION AND PHYLOGENY OF THE *ANOPHELES MINIMUS* COMPLEX (DIPTERA: CULICIDAE) INHABITING SOUTHEAST ASIAN COUNTRIES, BASED ON RIBOSOMAL DNA INTERNAL TRANSCRIBED SPACERS, ITS1 AND 2, AND THE 28S D3 SEQUENCES

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Abstract. *Anopheles minimus* (Theobald) is one of the most important vectors of human malaria in Southeast Asia. Morphological studies now have revealed five sibling species as its complex, designated as species A to E. The present study investigated the genetic divergence among *An. minimus* populations from four countries (Japan, China, Thailand and Indonesia), based on the DNA sequences data of the D3 (the third domain of the 28S ribosomal gene) and ITS2 (the second internal transcribed spacer of the ribosomal gene) is reported. The D3 and ITS2 phylogenetic trees, and the electrophoretic profile of ITS1 (the first internal transcribed spacer of the ribosomal gene) indicated that our *An. minimus* populations are comprised of three groups: the Japanese population as group I, the population from Guangxi Province of China (GX population) as group II, and others, as group III. The results showed the morphological similarity of group III and GX with the species complex A and B, respectively. It is interesting that both two species A (YN population) and species B (GX) occur in China, and that both species, *An. minimus* species A (LB-95 population) and the closer population *An. flavirostris* (Ludlow) (LB-00 population) appeared to be present on the Lombok Island of Indonesia, although in far separated localities. Moreover, this molecular evidence confirms the previous suggestion that the population from the Ishigaki Island of Japan should be classified as a new genetic status species E.

INTRODUCTION

Anopheles minimus (Theobald) is one of the most important vectors of human malaria in East Asian countries. It is found in India, Nepal, Bangladesh, Thailand, Indonesia, south China and also in the Yaeyama Islands of Japan. Based on the morphological study, *An. minimus* complex has now revealed five sibling species (species A and C; Sucharit *et al.*, 1988; Green *et al.*, 1990, form B; Sucharit *et al.*, 1988, form D; Baimai 1989, species E; Somboon *et al.*, 2001). Species A has been obtained from India, A and C from Vietnam, A, C and D from Thailand and A, B and

C from China (Subbarao, 1998) until now. It has been suggested that the prevalence of malaria infection could be associated with the morphological variations of the mosquitos inhabiting the infected areas (Yu and Li, 1984). Based on morphology of *An. minimus*, members of the complex are difficult to distinguish from closely related species, because they have overlapping morphological characters (Harrison, 1980), and *An. minimus* is a complex of at least two species, which are isomorphic and can only be distinguished using molecular markers.

Variations in protein polymorphism were helpful for establishing a genetic identity between the morphological species A and C identified in Thailand (Sucharit *et al.*, 1988; Green *et al.*, 1990), Vietnam (Van Bortal *et al.*, 1999), between species A and form B in China (Sawabe *et al.*, 1996) and for species E in the Yaeyama Islands (Sawabe, unpublished). Green *et al.* (1990) indicating that

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the electrophoretic band patterns of the allele of Octanol dehydrogenase (Odh) can be used to distinguish between species A and C. In molecular phylogenetic studies using DNA sequences, Sharpe *et al.* (1999) developed two polymerase chain reaction (PCR)-based methods for identifying species A and C, and other closely related species at the 3rd domain (D3) of the 28S gene of ribosomal DNA (rDNA) using PCR. Van Bortel *et al.* (2000) reported distinguishing species at the second internal transcribed spacer (ITS2) of the ribosomal gene.

In the previous study (Sawabe *et al.*, 1996), the genetic differentiation between the two *An. minimus* complex species inhabiting the Yunnan and Guangxi Provinces of China corresponds to the species A and form B, respectively. We thus report here the molecular variations and confirm genetically the status between them, and also for populations from three other Southeast Asian countries based on D3 and ITS2 sequences, and ITS1 electrophoretic profiles. Although Somboon *et al.* (2001) reported that the population from the Ishigaki Island of Japan should be classified as a new species E using the ISG population collected in 1999, we attempt to clarify their genetic background and also discuss the genetic identification of other ISG populations including the specimens used by Somboon *et al.* (2001).

MATERIALS AND METHODS

Mosquitos used

The details of all mosquitos used are given in Table 1. Ten *An. minimus* populations and one *An. flavirostris* were collected from four Southeast Asian countries. Morphological identification was performed on all individuals in the field for larvae and/or adults. Some populations were colonized and maintained at the laboratory after collection, and larvae at the end of the 4th-instar of the following generation were used for analysis. Based on the morphological characteristics and/or enzyme electrophoretic analysis, GX was confirmed as species B, ISG-93 and RKU as different from species A to D, and ISG-99 (namely ISG by Somboon *et al.*, 2001) as species E. All other populations were confirmed as species A. Details of the ISG-99 and the CMU were also

described in Somboon *et al.* (2001). All specimens were stored at -80°C until used.

For the DNA sequencing, male (CMU, LB-00, LMP, ISG-99), female adults (fraviro-00) and larvae (GX, ISG-93, LB-95, PK, RKU and YN) were used. The sequences of species A (minimus A, AF114019 and AF230461 for the D3 and the ITS2, respectively), species C (minimus C, AF114017 and AF230462 for the D3 and the ITS2, respectively) and two *Myzomyia* Series, *An. flavirostris* (*flavirostris*, AF194483 for the D3) and *An. aconitus* (Dönitz) (*aconitus*, AF114015 and AF194491 for the D3 and the ITS2, respectively), were obtained from GenBank database as references.

DNA extraction, amplification and sequencing

Genomic DNA was extracted from individual mosquitos using an IsoQuick DNA extraction Kit (Tanehashi, Tokyo, Japan). The regions of the D3, ITS1 and ITS2 were amplified in a final volume of 25 µl containing RTG PCR Beads (Amersham Pharmacia Biotech, Uppsala, Sweden) and primers using PCR (Model 9600, PE Applied Biosystems, USA).

The PCR cycling conditions were as follows: 30 seconds 95°C, 30 seconds 55°C and 1 minute 72°C for 35 cycles followed by a 4 minutes final extension at 72°C (for D3 primers); initial denaturation at 95°C for 5 minutes followed by 1 minute 95°C, 1 minute 39°C and 2 minutes 72°C for 45 cycles (for ITS1 and ITS2 primers). Primers used were 5'-GAC CCG TCT TGAAACACG GA-3' and 5'-TCG GAA GGA ACC AGC TAC TA-3' (Litvaitis *et al.*, 1994) for the D3 region, 5'-CCT TTG TAC ACA CGC CCC GT-3' and 5'-GTT CAT GTG TCC TGC AGT TCA C-3' (Sharpe *et al.*, 2000) for the ITS1 region, and 5'-TGT GAA CTG CAG GAC ACA T-3' and 5'-TAT GCT TAA ATT CAG GGG GT-3' (Beebe and Saul, 1995) for the ITS2 region, as a forward and reverse primer, respectively.

A five-µl portion of the PCR product was electrophoresed on a 2% agarose gel (NU Sieve 3:1, FMS Bio Product, USA), and the remainder was concentrated using ethanol precipitation methods. An excising individual band was cut away from a 2% LMP agarose (SeaPlaque GTG

agarose, USA) visualized by ethidium bromide, and cleaned using the methods for phenol-chloroform-ethanol purification. Each purified double-stranded PCR product was directly cycle-sequenced from both ends using Dye Terminator Cycle Sequencing FS Ready Reaction Kit (PE Applied Biosystems) and the same primers used for PCR using the thermal profile 10 seconds 96°C, 5 seconds 55°C and 4 minutes 60°C for 25 cycles (Model 9600, PE Applied Biosystems), and ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems).

Phylogenetic analysis

Alignment analyses were done using the program GENETYX-MAC ver. 10.01 (Software Development Co, Tokyo, Japan). Phylogenetic analysis was performed using distance and parsimony methods in MEGA (ver. 2.1; Kumar *et al*, 2001). Alignment gaps were treated as missing data. For the trees, a distance matrix was constructed using the Kimura 2-parameter model and trees were inferred using the neighbor-joining

method. The parsimony trees also were constructed using the heuristic search of maximum parsimony method. Because of these two phylogenetic trees inferred by different algorithms mentioned above showed similar topology, the numbers of bootstrap replicates from parsimony trees were added but not to drown the trees in the text. As no great differences were found within a population, one or two (for LMP) typical alignments were used for the following analyses and shown in this text.

RESULTS

D3 and ITS2 sequence alignments

Following electrophoresis of the PCR products through a 2% agarose gel, similar sized PCR products of ca. 380 bp were visible at D3 regions among all *An. minimus* populations. The alignment of the sequences, including those in minimus A, minimus C, and two *Myzomyia* Series mosquitos of *An. flavirostris* (namely flavirostris) and *An. aconitus* (namely aconitus) all from the

Table 1
Abbreviations and origins of individuals sequenced and GenBank accession numbers.

Species	Abbreviations	Geographical origin ^a	Regions		
			D3	ITS1	ITS2
<i>Anopheles minimus</i>	ISG-93	Nishihama-kawa, Ishigaki Is, Japan, 1993.11	3	3	3
	ISG-99	Nishihama-kawa, Ishigaki Is, Japan, 1999.8	5	5	5
	RKU ^b	Ryukyu University, Japan, 1992.11	-	-	5
	YN	Sumao, Yunnan Prov, China, 1993.8	3	3	3
	GX	Baise, Guangxi Prov, China, 1993.8	3	3	3
	PK	Pakalo, Mae Hong Son, Thailand, 1997.1	5	5	5
	LMP	Bantae, Lampang Prov, Thailand, 2000.12	3	3	3
	CMU ^c	Chiang Mai University, Thailand	8	8	8
	LB-95	Obel-Obel, Lombok Is, Indonesia, 1995.5	5	5	5
	LB-00	Labhan Poh, Lombok Is, Indonesia, 2000.1	5	5	5
	minimus A ^d	Kanchanaburi Prov, Thailand	AF114019	-	AF230461
	minimus C ^d	Kanchanaburi Prov, Thailand	AF114017	-	AF230462
<i>An. flavirostris</i>	flaviro-00	Labhan Poh, Lombok Is, Indonesia, 2000.1	2	2	2
	flavirostris ^d	Banggi Is. Sabah, Malaysia	AF194483	-	-
<i>An. aconitus</i>	aconitus ^d	Kanchanaburi Prov, Thailand	AF114015	-	AF194491

^aEach species was originally collected from its locality during 1993-2000;

Morphological identification was performed in the field;

^bRKU was colonized and maintained in the laboratory after it was collected in 1992 at the same locality of other two ISG populations;

^cCMU was colonized and maintained for more than 10 years in the laboratory.

^dThe sequences were obtained from GenBank database as references.

minimus A*	1:AAGCCAAT-GGGTAAATGGTGCGGTACGCCGCCCATGACTGGAACCACAGGCGAAGACAAATCGAGTGGTGCGGGATT
YN	1:.C.....-.....T.....
GX	1:GG.TT. AAA.....T.....AC.....T.....
PK	1:.....A.....
LMP1	1:.....-.....
LMP2	1:.....-.....
CMU	1:GGC..C.....
LB-95	1:CCC.....-.....T.....
LB-00	1:.....-.....T.....C.....C.....T.A.....
ISG-93	1:.GC.....G.....T.....T.....A.....T.....
ISG-99	1:..C.....-.....T.....A.....T.....
minimus C*	1:.....-.....AC.....T.....
flaviro-00	1:.....-.....T.....C.....T.A.....
flavirostris*	1:.....-.....T.....C.....T.A.....
aconitus*	1:.....-.....T.....T-GA.....T.....T.....
	* *
minimus A*	81:ACGGGTACGGCCGATGGCGCAAGCCTTCGTCGGACCCTCCATCCCAGGG-TGT-CCCCT--CCGGGTGCTTGACCCAG
YN	81:.....-.....-.....-.....
GX	81:.....-.....-.....-.....T.....
PK	81:.....-.....-.....-.....-.....-.....
LMP1	81:.....-.....-.....-.....-.....
LMP2	81:.....-.....-.....-.....-.....
CMU	81:.....-.....-.....-.....-.....
LB-95	81:.....-.....-.....-.....-.....
LB-00	81:.....T......G.....-.....C.G..CCAT.....
ISG-93	81:.....-.....-.....-.....-.....
ISG-99	81:..A.....-.....-.....-.....
minimus C*	81:.....-.....-.....-.....-.....T.....
flaviro-00	81:.....T......G......T..C.G..CCAT.....
flavirostris*	81:.....T......G......C.G..CCAT.....
aconitus*	81:.....T......G.....-.....T.....-TA.....
	* *
minimus A*	161:TGGACATCCCCGGAGTCGTAGGATGTGACC CGAAAGATGGTGAACATATGCCTGATCAGGTTGAAGTCAGGGGAAACCCT
YN	161:.....A.....
GX	161:C.....
PK	161:.....C.....C.....
LMP1	161:.....
LMP2	161:.....
CMU	161:.....
LB-95	161:.....
LB-00	161:..G.....
ISG-93	161:C.....
ISG-99	161:C.....
minimus C*	161:C.....
flaviro-00	161:..G.....
flavirostris*	161:..G.....
aconitus*	161:.....T.....C.....
	* *
minimus A*	241:GATGGAGGACC GAAGCAATTCAGCGTGCAAATCGATTGTCAGAGTTGGGCATAGGGCGAAAGACC
YN	241:.....T----G.....
GX	241:.....CTT...--.....A.....
PK	241:.....G.....
LMP1	241:.....G.....
LMP2	241:.....GG.....
CMU	241:.....G..C.....
LB-95	241:.....TT.....-.....C.....
LB-00	241:.....-.....A.....A.....
ISG-93	241:.....TATA.-G..C.....
ISG-99	241:.....G.....G..C.....
minimus C*	241:.....
flaviro-00	241:.....T.....-.....-G.....
flavirostris*	241:.....
aconitus*	241:.....
	* *

Fig 1–Nucleotide sequences of nuclear ribosomal D3 region of 28S gene. Dots denote homology with the minimus A sequence (GenBank accession number AF114019). Populations marked with asterisks indicate populations obtained from the GenBank database. For explanation of abbreviations of species names, see Table 1.

Table 2

Percentage pairwise differences among D3 (above diagonal) and ITS2 (below diagonal) sequences.

Species		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
<i>An. minimus</i>	1 ISG-93		2.7		4.1	5.2	5.9	4.8	5.2	3.7	3.8	8.1	4.5	4.1	7.4	7.3	7.4
	2 ISG-99	1.6			4.1	6.3	4.1	3.4	3.7	3.0	4.4	6.6	3.0	2.7	6.3	5.9	6.7
	3 RKU	1.0	1.6														
	4 YN	4.6	4.8	3.7		5.6	2.4	2.0	2.4	2.7	2.0	6.3	1.7	3.4	6.0	5.2	6.0
	5 GX	4.6	5.4	4.8	5.4		6.6	5.9	6.3	5.9	5.6	9.3	5.5	3.8	8.6	8.1	8.2
	6 PK	3.5	4.6	2.9	1.0	4.8		0.7	1.0	3.0	3.7	5.5	1.0	2.7	5.2	4.4	5.2
	7 LMP1	4.3	4.8	3.7	1.3	5.1	0.8		0.3	2.4	3.1	4.8	0.3	2.0	4.5	3.7	4.5
	8 LMP2	4.8	4.8	4.8	2.4	6.2	1.8	1.3		2.7	3.4	5.2	0.7	2.4	4.1	4.1	4.9
	9 CMU	4.8	5.4	4.3	1.8	5.7	1.3	0.5	1.3		2.7	6.2	2.0	3.7	5.9	5.5	6.3
	10 LB-95	4.0	4.6	3.5	1.0	4.8	0.5	0.8	1.8	0.8		7.0	2.7	4.4	6.7	6.2	6.3
	11 LB-00	8.5	9.7	8.0	8.8	8.5	8.0	8.8	10.0	9.4	8.5		4.4	5.2	1.3	1.0	5.6
	12 minimus A*	4.0	4.6	3.5	0.5	4.8	0.5	0.8	1.8	1.3	0.5	8.5		1.7	4.1	3.4	4.1
	13 minimus C*	4.8	5.7	4.6	4.6	0.8	4.6	4.8	6.0	5.4	4.6	8.8	4.0		4.8	4.1	4.9
<i>An. flavirostris</i>	14 flaviro-00	9.4	10.0	8.8	9.1	8.8	8.8	9.1	9.7	9.1	8.8	0.8	8.8	9.1		0.7	5.6
	15 flavirostris*																4.8
<i>An. aconitus</i>	16 aconitus*	22.6	23.3	21.9	20.9	19.9	21.2	21.6	22.6	22.3	21.2	16.5	20.5	19.5	16.9		

Values are transitions+transversions. Values are calculated from 307bp and 479bp in D3 and ITS2 sequences, respectively.

GenBank database, were compared at 307 bp of their lengths (Fig 1). Both ends of the alignments had some variations, but coincided in the intermediate regions. In the case of ITS2 sequences, lengths of approximately 480 bp were amplified among all populations. As shown in Fig 2, alignments of 479 bp in length showed several insertions and deletions (423-478 bp). Many more variations were found in ITS2 than in D3.

Nucleotide variations at D3 and ITS2 regions

The levels of nucleotide variation detected between pairs of specimens for D3 and for ITS2 are presented in Table 2. Percentage nucleotide differences within ISG populations were 2.7% (between ISG-93 and ISG-99) for the D3 region, and 1.0-1.6% for the ITS2 region, respectively. The ISG populations showed higher differences from the other *An. minimus* populations, except for the LB-00 (2.7-6.6% for D3, 2.9-5.7% for ITS2). Among all of the *An. minimus* populations, except for the LB-00, the differences were very low ranging 0.3-6.6% for the D3 and 0.5-6.2% for the ITS2. The differences between the LB-00 and two *An. flavirostris* populations (flaviro-00

and flavirostris) were 1.0% and 1.3% for the D3 and 0.8% for the ITS2. These values showed markedly lower levels of genetic differences.

Phylogenetic trees

The neighbor-joining trees inferred from the sequence data of the D3 and the ITS2 regions are shown in Figs 3 and 4, respectively. From the D3 sequences (Fig 3), the *An. minimus* populations could be separated into three clusters: the Japanese population (ISG-93 and ISG-99) as group I, the GX from China and minimus C as group II, and the others (PK, LMP1 and 2, CMU, YN, LB-95) including minimus A as group III. *Anopheles flavirostris* and *An. aconitus* were widely separated from the *An. minimus* complex. They are suggested to represent an ideal outgroup of the *An. minimus* populations. The LB-00 was closer to this group than the *An. minimus* group. These classification results from the ITS2 sequences (Fig 4) were similar to the D3 sequences. The RKU was included in the Japanese population clustered by ISG-93 and ISG-99. The ITS2 sequences also indicated that LB-00 was closer to *An. flavirostris* than to the *An. minimus* group, although LB-95

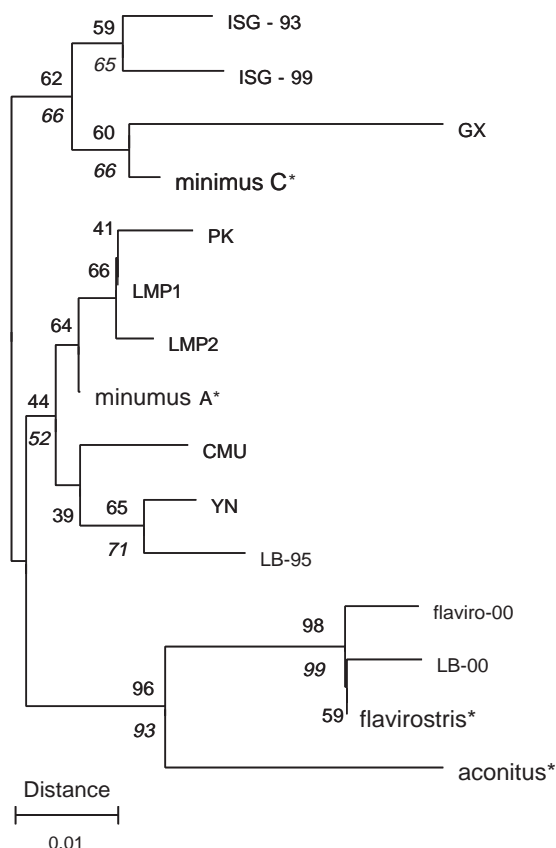


Fig 3—Phylogenetic tree of *An. minimus* complex inferred from the D3 sequences shown in Fig 1. For explanations of abbreviations of species names, see Table 1. Tree was constructed using the neighbor-joining method in the MEGA ver. 2.1, and a distance matrix was calculated using the Kimura's 2-parameter evolutionary model. Numbers at above nodes are percentage values of 1,000 bootstrap replicates with neighbor-joining tree, and numbers in italic at below nodes are with maximum parsimony tree in the MEGA ver. 2.1. The scale bar indicates the estimated genetic distance (percent).

was included in the *An. minimus* group.

ITS1 electrophoretic profiles

The ITS1 region was abandoned because of the large size of the spacer (ca. 1,500 bp) and the presence of a repeat structure. It was anticipated that the complete sequence could not be amplified by one PCR trial. However, some minor bands were found on the 2% agarose gel (Fig 5),

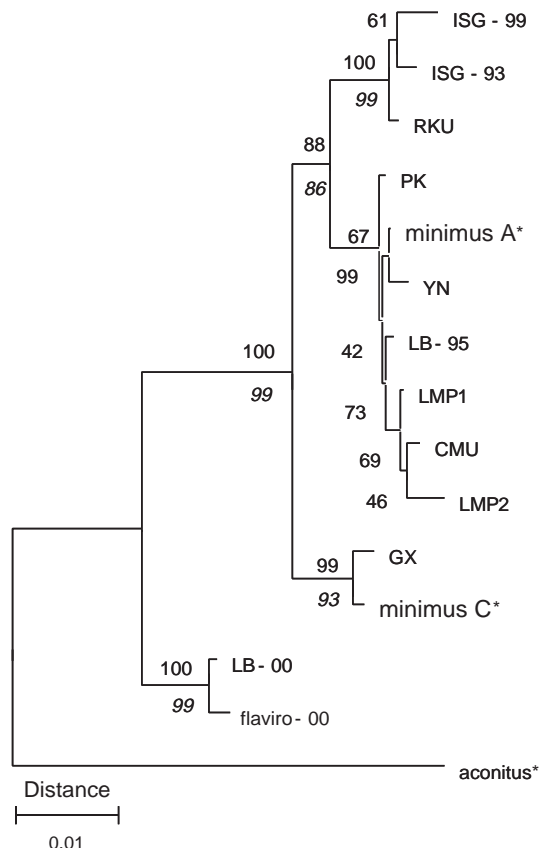


Fig 4—Phylogenetic trees of *An. minimus* complex inferred from nuclear ribosomal ITS2 nucleotide sequences shown in Fig 2. For explanations of abbreviations of species names, see Table 1. Tree was constructed using the neighbor-joining method in the MEGA ver. 2.1, and a distance matrix was calculated using the Kimura's 2-parameter evolutionary model. Numbers at above nodes are percentage values of 1,000 bootstrap replicates with neighbor-joining tree, and numbers in italic at below nodes are with maximum parsimony tree in the MEGA ver. 2.1. The scale bar indicates the estimated genetic distance (percent).

and all individuals per locality showed same electrophoretic band patterns. They could be classified into three groups as shown in the above-mentioned D3 and ITS2 sequences by their patterns.

DISCUSSION

In the present study, we inferred the sequence differences in the nuclear DNA at the D3 and the

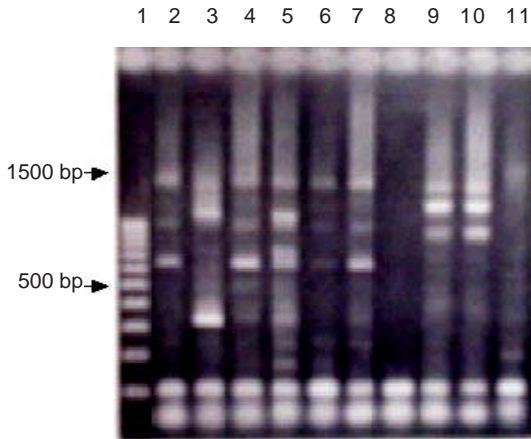


Fig 5—An ethidium bromide-stained 2% agarose gel showing the PCR products spanning the nuclear ribosomal ITS1 region. Lane 1: 100-bp ladder; lane 2: YN, lane 3: GX, lanes 4: PK, lane 5: LMP, lane 6: CMU, lanes 7: LB-95, lane 8: LB-00, lane 9: ISG-93, lane 10: ISG-00, lane 11: flaviro-00.

ITS2 regions among populations of the *An. minimus* complex collected from four Southeast Asian countries. Phylogenies indicated that they could be clustered into three groups: three ISG populations as group I, the GX as group II, and the others (YN, CMU, PK, LMP, LB-95) as group III, in which *minimus* A is included. This result agrees well with the morphological classification that members of group III are species A, and those from group II (GX), are species B (unpublished data). In this phylogenetic tree, the ISG populations presented in the cluster are separate from *minimus* A, B and C (GenBank accession numbers AF114017 and AF230462). Somboon *et al* (2001) reported that the ISG population (ISG-99 in the present study) should be classified as a new species, *An. minimus* species E, based on the results from crossing experiments between CMU and ISG populations and on the morphological characteristics. For the species D, although there is no molecular information but morphological data (Baimai, 1989), we demonstrated that the other two ISG populations (ISG-93 and RKU) should probably be classified as species E.

Anopheles minimus is widespread in Southeast Asian countries. In Japan, this species is now

localized in the Southern Islands, and no endemic malaria has been reported since 1962 (WHO, 1966). The distribution of many animals and insects gradually changing and the characteristics of the various species of malaria vector mosquitoes may also be changing. An ecological survey of these areas has been carried out periodically (Toma *et al*, 1996a, Tsuda *et al*, 1999, 2000). The species has not been clarified either morphologically or by a protein polymorphism study (KS, unpublished data). Yu *et al* (1984) suggested that the prevalence of malaria infection could be associated with its morphological variant species of mosquito inhabiting the endemic areas of China. In Northern Vietnam, the two sibling species differ in some behavioral traits that are relevant for malaria transmission and vector control (Van Bortel *et al*, 2000). As they inferred, the relationship between the sensitivity for malaria transmission and the morphological variants of vector mosquito is of a great interest to us. Further investigation on these Japanese populations should be performed as soon as possible to clarify the genetic background and to estimate the sensitivity of transmission of the malaria parasite.

The Chinese species B (GX) is the closest to the Thai species C (*minimus* C) rather than Chinese species A (YN). The GX constructs a cluster in both the D3 and ITS2 phylogenetic trees, however, the branch length in the D3 tree is longer than in the ITS2 tree. This indicates that the rate of evolution of GX has increased since diverging from *minimus* C. The D3 phylogenetic tree is drawn as an unrooted one, contrary to the ITS2 tree rooted in *An. aconitus*. The D3 primers (Litvaitis *et al*, 1994) used in this study were designated as being able to distinguish species C from A. This suggests that the D3 primers are much more specific for identification of closely related cryptic species such as species A and C. The ITS2 sequences are relatively conserved and intraspecific variation is minimal or non-existent in trematodes (Morgan and Blair, 1995; Despres *et al*, 1992). Actual divergences among species complexes could appear for the *An. minimus* group.

In the present study, the LB-95 was an in-group of species A, but LB-00 was not and closer to *An. flavirostris* than to any other *An. minimus* populations. It is interesting that both species, *An.*

minimus species A (LB-95) and the closer population to *An. flavirostris* (LB-00) appeared to be present on Lombok Island in Indonesia, although in far separated localities. The former was collected from Obel-Obel on Lombok Island in May 1995, and the latter from Labhan Poh on Lombok Island in January 2000. These two populations inhabited different streams that are located at opposite ends of the Island. The difference between LB-95 and LB-00 could be caused by annual, seasonal and/or a regional prevalence of the mosquito. Somboon *et al* (2000) described in the paper that *An. flavirostris* was found in Indonesia, but *An. minimus* was not. Among Myzomyia Series, such as *An. flavirostris*, *An. aconitus* and *An. minimus*, there are no great differences in the morphological characteristics. Cross inhabiting in the same locality is conceivable. Our results suggest that additional ecological and genetic surveys are needed on Lombok Island.

The alignments of the ITS1 and ITS2 regions are generally discussed for the intraspecific variation between closely related sibling species. Sharpe *et al* (2000) did not use the ITS1 data in their phylogenetic analysis of the *An. minimus* group from Thailand, because of the presence of a repeat structure. The ITS1 region was abandoned because of the large size of the spacer (ca. 1,500 bp). The sequence data from the ITS1 region was not clearly obtained in our study either, but an electrophoretic profile from the PCR product was detected on the agarose gel. The results from the ITS1 profile was nearly equal to the classifying trend of the D3 and ITS2 phylogenies. Van Bortel *et al* (2000) obtained clear diagnostic banding patterns of the ITS2 rDNA fragment from six *An. minimus* related species on agarose gels after digestion with two kinds of restriction endonuclease. Our banding patterns for the ITS1 PCR product would be a useful diagnostic tool without sequencing for this species complex. Further examination using multiple numbers of complexes species and specimens from the species are needed in the future. The mitochondrial DNA can be a sensitive marker of phylogenetic relationship between cryptic species complexes at the genetic level for many kinds of organisms (Nei, 1983). Data from the mitochondrial COII region would be useful for our phylogenetic study.

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REFERENCES

- Baimai V. Speciation and species complexes of the *Anopheles* malaria vectors in Thailand. In: Proceedings of the 3rd Conference on Malaria Research, Thailand. 1989: 146-62.
- Beebe NW, Saul A. Discrimination of all members of the *Anopheles punctulatus* complex by polymerase chain reaction restriction fragment length polymorphism analysis. *Am J Trop Med Hyg* 1995; 53: 478-81.
- Despres L, Imbert-Establet D, Combes C, Bonhomme F. Molecular evidence linking hominid evolution to recent radiation of Schistosomes (Platyhelminthes: Trematoda). *Mol Phylogenet Evol* 1992; 1: 295-304.
- Green CA, Gass RF, Munstermann LE, Baimai V. Population-genetic evidence for two species in *Anopheles minimus* in Thailand. *Med Vet Entomol* 1990; 4: 25-34.
- Harison BA. Medical entomology studies. XIII. The Myzomyia Series of *Anopheles (Cellia)* in Thailand, with emphasis on Intra- and interspecific variations (Diptera: Culicidae). *Contrib Am Entomol Inst (Ann Arbor)* 1980; 17: 1-195.
- Kumar S, Tamura K, Jakobsen IB, Nei M. MEGA2: Molecular Evolutionary Genetics Analysis software, ver. 2.1, Arizona State University, Tempe, Arizona, USA, 2001.
- Litvaitis MK, Nunn G, Thomas WK, Kocher TD. A molecular approach for the identification of *Meiofaunal turbellarians* (Platyhelminthes, Turbellaria). *Marine Biol* 1994; 120: 437-42.
- Morgan JA, Blair D. Nuclear rDNA ITS sequence variation in the trematode genus *Echinostoma*: an

- aid to establishing relationships within the 37-collar-spine group. *Parasitology* 1995; 111: 609-15.
- Nei M. Genetic polymorphism and the role of mutation in evolution. In: Nei M, Koehn R, eds. *Evolution of genes and proteins*. Sunderland, Mass: Sinauer Associates, 1983: 165-90.
- Sawabe K, Takagi M, Tsuda Y, *et al*. Genetic differentiation among three populations of *Anopheles minimus* mosquitoes of Guangxi and Yunnan Provinces in the Peoples Republic of China. *Southeast Asian J Trop Med Public Health* 1996; 27: 818-7.
- Sharpe RG, Hims MM, Harbach RE, Butlin RK. PCR based methods for identification of species of the *Anopheles minimus* group: allele specific amplification and single strand conformation polymorphism. *Med Vet Entomol* 1999; 13: 265-73.
- Sharpe RG, Harbach RE, Butlin RK. Molecular variation and phylogeny of members of the *Minimus* group of *Anopheles* subgenus *Cellia* (Diptera: Culicidae). *Syst Entomol* 2000; 25: 263-72.
- Somboon P, Tuno N, Tsuda Y, Takagi M. Evidence of the species status of *Anopheles flavirostris* (Diptera: Culicidae). *J Med Entomol* 2000; 37: 476-9.
- Somboon P, Walton C, Sharpe RG, *et al*. Evidence for a new sibling species of *Anopheles minimus* from the Ryukyu Archipelago, Japan. *J Am Mosq Control Assoc* 2001; 17: 98-113.
- Subbarao SK. Anopheline species complexes in South-East Asia. *WHO Techn Pub SEARO* 1998; 18: 46-9.
- Sucharit S, Komalamisra N, Leemingsawat S, Apiwathnasorn C, Thongrungrat S. Population genetic studies on the *Anopheles minimus* complex in Thailand. *Southeast Asian J Trop Med Public Health* 1988; 19: 717-23.
- Toma T, Miyagi I, Malenganisho WL, *et al*. Distribution and seasonal prevalence of the malaria vector mosquito, *Anopheles minimus*, in Ishigaki Is., Ryukyu Archipelago, Japan, 1990-1994. *Med Entomol Zool* 1996a; 47: 63-72.
- Toma T, Miyagi I, Takagi M, Tsuda Y. Survey of *Anopheles minimus* in Miyako Island, Ryukyu Archipelago, Japan, 1991 and 1995. *Med Entomol Zool* 1996b; 47: 167-70.
- Tsuda Y, Takagi M, Suwonkerd W. A mark-release-recapture study on the spatial distribution of host-seeking *Anopheles* in northern Thailand. *J Vector Ecol* 2000; 25: 16-22.
- Tsuda Y, Takagi M, Toma T, Sugiyama A, Miyagi I. Mark-release-recapture experiment with adult *Anopheles minimus* (Diptera: Culicidae) on Ishigaki Island, ryukyu Archipelago, Japan. *J Med Entomol* 1999; 36: 601-4.
- Van Bortel W, Trung HD, Manh ND, Roelants P, Verle P, Coosemans M. Identification of two species within the *Anopheles minimus* complex in northern Vietnam and their behavioral divergences. *Trop Med Int Health* 1999; 4: 257-65.
- Van Bortel W, Trung HD, Roelants P, Harbach RE, Backeljau T, Coosemans M. Molecular identification of *Anopheles minimus* s.l. beyond distinguishing the members of the species complex. *Insect Mol Biol* 2000; 9: 335-40.
- World Health Organization (WHO). Report of the WHO evaluation team on malaria eradication in the Ryukyu Islands Geneva, Switzerland: *WHO WPR/419/1966*; 65: 1-53.
- Yu Y, Li M. Notes on the two forms of *Anopheles (Celia) minimus* Theobald, 1901 in Hainan Island. *J Parasitol Parasit Dis* 1984; 2: 95-8 (in Chinese with English summary).
- Yuan Y. Studies on the two forms of *Anopheles (Cellia) minimus* Theobald, 1901 in China (Diptera: Culicidae). *Mosq Syst* 1987; 19: 143-5.