SEQUENCE ANALYSES OF THREE NUCLEAR RIBOSOMAL LOCI AND A MITOCHONDRIAL LOCUS IN CYTOLOGICALLY DIFFERENT FORMS OF THAI *ANOPHELES ACONITUS* MOSQUITOES

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Abstract. Ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) of *Anopheles aconitus* mosquitoes were examined to investigate intra- and inter-species variation amongst the members of the Minimus group of *Anopheles* subgenus *Cellia*. Three rDNA loci (ITS1, ITS2 and D3 regions) and a mtDNA locus (cytochrome oxidase II) were analyzed in *An. aconitus* Form B and Form C collected in Chiang Mai Province, Thailand. The results show that the consensus sequences of the four loci of the two forms are consistent with those of mosquitoes in the genus *Anopheles*. No intraindividual variation was detected, but intrapopulation variation was present with polymorphic sequences in some forms for each gene examined. The variation rates were approximately 0.15 to 0.8%. These data indicate that *An. aconitus* Form B and Form C in Chiang Mai, Thailand are conspecific. In this study, the complete ITS1 sequence of *An. aconitus* is reported for the first time. The region showed a high variation rate (approximately 55%), compared to the closely related species *An. minimus* C. It is suggested that this rDNA locus may provide sequence information to differentiate the members of the Minimus group of *Anopheles* subgenus *Cellia*.

INTRODUCTION

Ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) of mosquitoes are useful for studying genetic variability and divergences within and among species (Norris, 2002). The rDNA consists of tandemly repeated transcriptional units with highly conserved genes, and occurs with approximately 500 copies in the genome of mosquitoes (Collins *et al*, 1987). Within each transcriptional unit, spacers separate the 18S, 5.8S and 28S rDNA subunits, the internal transcribed spacer 1 (ITS1) and 2 (ITS2), respectively, and between two transcriptional units, there is non-transcribed region called the intergenic spacer (IGS). Because of the relatively rapid rate at which new mutants are fixed in the rDNA spacers, these regions have become popular targets for addressing taxonomic issues among anophelines. As the number of recognized anopheline species complexes grows, sequence information of the spacers is developed into molecular diagnostic tools (PCR-based diagnostic, RFLP-based diagnostic and DNA hybridization) that differentiate between cryptic taxa. Among these spacers the ITS2 has been extensively used for the differentiation of species within the *An. funestus* group (Hackett *et al*, 2000), *An. punctulatus* group (Beebe and Saul, 1995), *An. minimus* group (Van Bortel *et al*, 2000; Phuc *et al*, 2003), *An. maculipennis* complex (Porter and Collins, 1996), *An. dirus* complex (Xu *et al*, 1998; Walton *et al*, 1999), *An. fluviatilis* complex (Manonmani *et al*, 2001), and *An. quadrimaculatus* complex (Cornel *et al*, 1996, 1997). The IGS has been used to distinguish the members of the *An. gambiae* complex (Scott *et al*, 1993). Although not as widely used

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as ITS2 in mosquitoes, ITS1 has similar properties to ITS2 and has been used at the population level to study the hard tick *Ixodes scapularis* (McLain *et al*, 1995).

Mitochondrial DNA has also been similarly utilized in investigations of a wide variety of anophelines and anopheline species complexes. Frequent targets used in phylogenetic and population genetic studies include both coding regions, for example, NADH dehydrogenase subunit 5 (*ND5*) and cytochrome oxidase subunits I and II (*COI* and *COII*), and non-coding regions, such as 16S and 12S RNA genes. Foley *et al* (1998) used the *COII* gene to derive the phylogeny of Australasian anophelines; de Merida *et al* (1999) used mtDNA (*ND5*) and single-strand conformation polymorphism (SSCP) analysis to examine the population structure of *An. albimanus*; and Fairley *et al* (2000) used a *COI* gene fragment to look at genetic structuring and gene flow among a population of *An. punctipennis* in Vermont. However, mtDNA has failed to differentiate cryptic taxa within the *An. maculipennis* complex (Collins *et al*, 1990).

The purpose of the current study is to apply these methods to *An. aconitus* in Thailand. Recently, Sharpe *et al* (2000) investigated intra- and inter-specific variation in four members of the Minimus group of *Anopheles* subgenus *Cellia*: *An. aconitus*, *An. varuna*, *An. minimus* A and C. They reconstructed phylogenetic relationships of this group and estimated divergence times between *An. minimus* A and C using data from one mitochondrial COII and one ribosomal nuclear (D3) locus. Sharpe *et al* (1999) also used allele-specific amplification of the D3 variable region of the 28S rDNA to distinguish *An. minimus* A from *An. minimus* C, and SSCP of the D3 amplified region to discriminate four species: *An. aconitus*, *An. varuna*, *An. minimus* A and C collected in Kanchanaburi, Tak and Chiang Mai Provinces, Thailand. In this study, two haplotypes (haplotype 1: 1 wild-caught female, Tak Province, 1 wild-caught female, Chiang Mai Province, 2 wild-caught females, Kanchanaburi Province; haplotype 2: 1 wild-caught female Kanchanaburi Province) of *An. aconitus,* were reported. These raise questions about the genetic divergence of *An. aconitus* in Thailand. In 1996, Baimai *et al* reported three karyotypic forms of *An. aconitus* from Mae Taeng district, Chiang Mai Province: Form A (X_1, X_2, Y_1), Form B (X_1, X_2, Y_2), and Form C (X_1, X_2, Y_3). Little is known about the molecular markers, including the rDNA and mtDNA, of the three forms. In the present study, three rDNA loci, ITS1, ITS2 and the D3 region of 28S rDNA, and one mtDNA locus, COII of cytologically different forms of *An. aconitus,* were described and compared for the first time. The sequence data extends our understanding of the mosquito species in Thailand and Southeast Asia.

MATERIALS AND METHODS

Sample collection and isofemale line establishment

Isofemale lines were established from wild-caught, fully engorged *An. aconitus* females collected from an endemic area of malaria, Ban Pang Mai Daeng, Mae Taeng district, Chiang Mai Province, Thailand, the area where Baimai *et al* (1996) incriminated the three karyotypic forms of *An. aconitus*, in October 2002-June 2004. Metaphase chromosomes were prepared from newly-emerged adult F_1 and/or F_2 progenies of each isoline (Choochote *et al*, 2001), and karyotypic forms were identified using the cytotaxonomic key of Baimai *et al* (1996). The mosquitoes were further colonized using the techniques described by Choochote *et al* (1983). One female from each identified isoline was used for DNA extraction.

DNA extraction, PCR amplification, cloning and sequencing

Genomic DNA was extracted from individual mosquitoes with a DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions. Primers for PCR amplification of the ITS1, ITS2, D3 and COII regions are listed in Table 1. High fidelity DNA polymerase, Platinum® *Taq* polymerase (Invitrogen) was used for PCR amplification. PCR was performed using the following conditions: one cycle of 4 minutes at 94ºC; 20 cycles of 1 minute at 94ºC, 50 seconds at 52ºC and 2 minutes at 72ºC; and a final cycle of 5 minutes at 72ºC. PCR products were cloned in PCR® 4-TOPO® plasmids, TA Cloning Kit for sequencing (Invitrogen)

following the manufacturer's instructions. Recombinant plasmids were purified using QIAGEN miniprep columns (Qiagen) before sequencing using an automated sequencing system at the BSU Bioservice Unit, National Science and Technology Development Agency (NSTDA) Building, Bangkok, Thailand. Two to three clones of each mosquito were sequenced.

DNA sequence analysis

DNA sequences were analyzed using the CLUSTAL W program (Higgins *et al*, 1996). Sequence data of *An. aconitus* rDNA and COII, and *An. minimus* C rDNA reported by Sharpe *et al* (2000) were retrieved from GenBank. Symbols and GenBank accession numbers are given in Table 2.

RESULTS

After 217 specimens were carefully identified for karyotypic forms, *An. aconitus* Form A (X_1, X_2, Y_1) was not found in this current study. One hundred and twenty-eight specimens were identified as *An. aconitus* Form B, and 89 specimens as Form C. Therefore, the ITS1, ITS2 and D3 regions of rDNA and COII of mtDNA were investigated in only two karyotypic forms of *An. aconitus*, Form B and Form C (Fig 1).

In individual mosquitoes, no clonal variation was observed in any sequences of each region.

Sequence analysis of ITS1

Two identical ITS1 sequences (BITS1) were obtained from 5 isolines of *An. aconitus* Form B and two others (CITS1) from 5 isolines of Form C. Sequence alignment for the ITS1 is given in Fig 2. The ITS1 sequences of both forms were 503 bp long and show over 99% identity. Their GC contents were approximately 48%. The boundaries of ITS1 were defined according to the previously published sequence for *An. minimus* C (Sharpe *et al,* 2000). The *An. aconitus* ITS1 shown in Fig 2 begins at position 184 and ends at position 686 of the amplified DNA, being flanked by approximately 183 bp of the 3′ end of the 18S, and 86 bp of the 5′ end of the 5.8S rDNA genes. Two base substitutions were detected at positions 306 and 329 of both loci (variation rate = 0.4%). Comparison of ITS1 DNA sequences among *An. aconitus* Form B, Form C and *An. minimus* C (Sharpe *et al*, 2000) was performed. Summary of the sequence variation is shown in Table 3. *An. minimus* C ITS1 sequences diverged more markly from *An. aconitus* Form B and Form C with 55.4% and 55.2% variation, respectively.

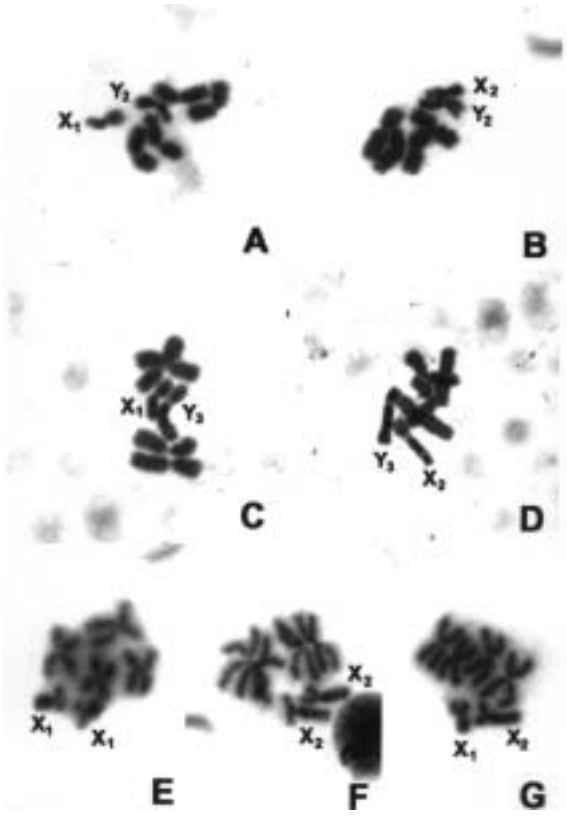

Fig 1–Metaphase karyotypes of *Anopheles aconitus* Forms B and C collected in Chiang Mai, Thailand (Giemsa staining). Testes chromosomes: (A) Form B; showing X_1,Y_2-chromosomes, (B) Form B; showing X_2,Y_2-chromosomes, (C) Form C; showing X_1,Y_3-chromosomes, (D) Form C; showing X_2,Y_3-chromosomes. Ovary chromosomes: (E) showing homozygous X_1,X_1-chromosomes, (F) showing homozygous X_2,X_2-chromosomes, (G) showing heterozygous X_1,X_2-chromosomes. Note, all types of X-chromosomes were found in all forms and strains of *An. aconitus.*

Sequence analysis of ITS2

DNA sequences of *An. aconitus* Form B and

Table 1
Primers used for PCR amplification. f = forward; r = reverse.

Primers	Sequence (5′ → 3′)	Priming region	References
ITS1			
18S (f)	CCTTTGTACACACCGCCCGT	18S	Sharpe *et al* (2000)
ITS6 (r)	GTTCATGTGTCCTGCAGTTCAC	5.8S	Sharpe *et al* (2000)
ITS2			
ITS2A (f)	TGTGAACTGCAGGACACAT	5.8S	Beebe and Saul (1995)
ITS2B (r)	TATGCTTAAATTCAGGGGGT	28S	Beebe and Saul (1995)
D3			
D3a (f)	GACCCGTCTTGAAACACGGA	28S	Sharpe *et al* (1999)
D3b (r)	TCGGAAGGAACCCGCTACTA	28S	Sharpe *et al* (1999)
COII			
LEU (f)	TCTAATATGGCAGATTAGTGCA	tRNA-LEU	Sharpe *et al* (2000)
LYS (r)	ACTTGCTTTCAGTCATCTAATG	tRNA-LYS	Sharpe *et al* (2000)

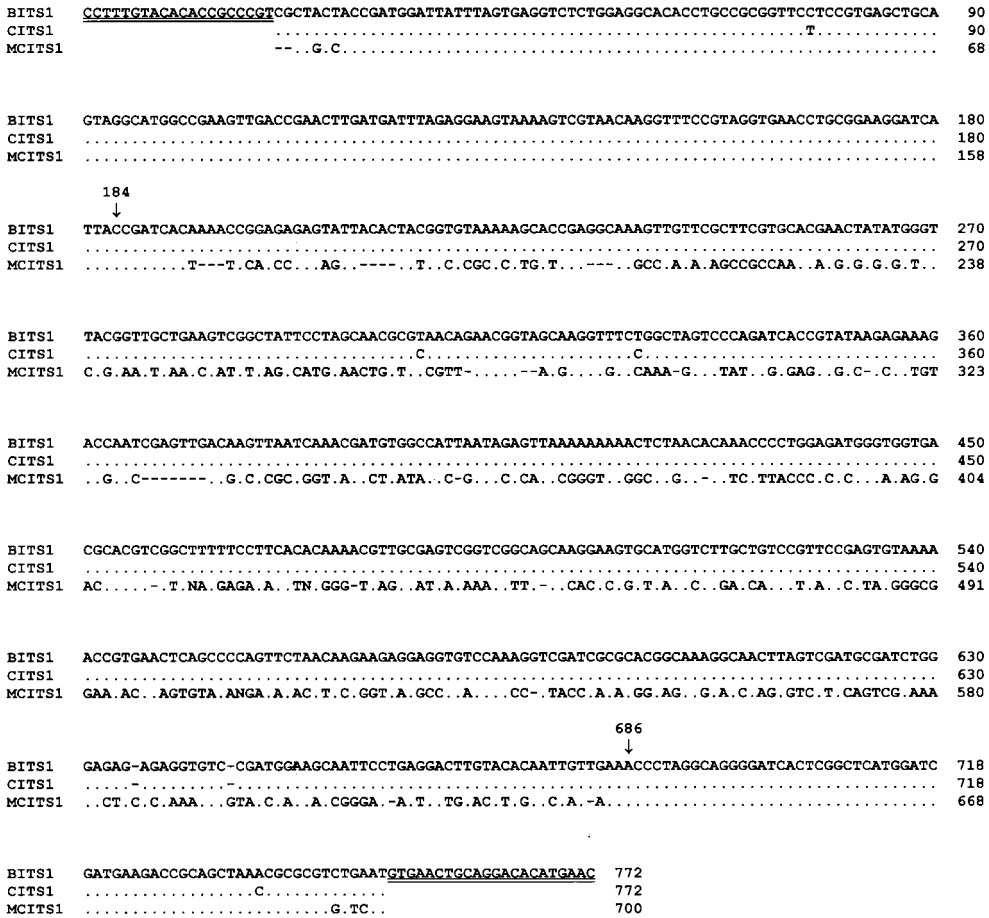

Fig 2–Alignment of the ITS1 and flanking 18S and 5.8S regions of rDNA for *Anopheles aconitus* Form B (BITS1), Form C (CITS1) and *An. minimus* C (MCITS1). Dots indicate sequence identity with BITS1; dashes represent gaps introduced to maximize overall sequence similarity. The target sequences for PCR primers that flank the region analyzed are double-underlined. Arrows at 184 and 686 delineate the ITS1. Sequence names are defined in Table 2.

Table 2
Taxa examined, their GenBank accession numbers and sources of specimens.
CM = Chiang Mai Province, northern Thailand; KB = Kanchanaburi province, central Thailand.

Region	Approx PCR product size (bp)	Number of individuals sequenced/species name/unique sequences identified	Symbol	GenBank accession number	Geographic origin	Reference
ITS1	772	2/*An. aconitus* Form B/1	BITS1	AY547356	CM	This study
	772	2/*An. aconitus* Form C/1	CITS1	AY547357	CM	This study
	-	1/*An. minimus* C/1	MCITS1	AF194480-1	KB	Sharpe *et al* (2000)
ITS2	517	9/*An. aconitus* Form B/4	BITS2-1,	AY547358	CM	This study
	517		BITS2-2,	AY547359	CM	
	517		BITS2-3,	AY547360	CM	
	515		BTIS2-4	AY547361	CM	
	517	3/*An. aconitus* Form C/2	CITS2-1,	AY547362	CM	This study
	516		CITS2-2	AY547363	CM	
	-	4/*An. aconitus*	UITS2-1,	AF194494	KB	Sharpe *et al* (2000)
	-	(unidentified form, U)/2	UITS2-2	AF194493	KB	
D3	375	5/*An. aconitus* Form B/2	BD3-1,	AY547364	CM	This study
	375		BD3-2	AY547365	CM	
	375	4/*An. aconitus* Form C/1	CD3	AY547366	CM	This study
	-	3/*An. aconitus*	UD3-1,	AF114015	KB	Sharpe *et al* (1999)
	-	(unidentified form, U)/2	UD3-2	AF114014	KB	
COII	770	4/*An. aconitus* Form B/2	BCOII-1,	AY547367	CM	This study
	770		BCOII-2	AY547368	CM	
	770	4/*An. aconitus* Form C/1	CCOII	AY547369	CM	This study
	-	4/*An. aconitus*	UCOII-1,	AF194448	KB	Sharpe *et al* (2000)
	-	(unidentified form, U)/2	UCOII-2	AF194451	KB	

Table 3
Summary of sequence variation of the ITS1 region of rDNA among *Anopheles aconitus* Form B, Form C and *An. minimus* C.

Species or forms (see symbol in Table 2)	Length (bp)	Length difference (bp)	Number of fixed nucleotide substitutions (%)	Number of fixed indel (%)	Total variation (%)
BITS1/ CITS1	503/503	0	2 (0.4)	0 (0)	0.4
BITS1/ MCITS1	503/474	29	264 (49.6)	31 (5.8)	55.4
CITS1/ MCITS1	503/474	29	263 (49.4)	31 (5.8)	55.2

Form C with approximately 515 to 517 bp in length were obtained from PCR amplification using ITS2A and ITS2B primers. The boundaries of the 5.8S and 28S regions were estimated by comparison with those determined by Sharpe *et al* (2000), for the Minimus group. Alignment for the ITS2 of *An. aconitus* Form B, Form C, and an unidentified form of *An. aconitus* reported by Sharpe *et al* (2000) is shown in Fig 3. The ITS2 of *An. aconitus* begins at position 96 and ends at position 473. The lengths of the ITS2 sequences were 376 to 378 bp. The sequences were slightly GC rich (56%). Simple tandem repeats were present at various locations along the ITS2. For example, CA was repeated at positions 109, 141 and 234, AG at positions 241 and 278, and CAT at position 403. Other tandem repeats of ACGCAT at position 434 and

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BITS2-1 TGTGAACTGCAGGACACATGAACACCGACACGTTGAACGCATATGGCGCATCGGACGTTTAAACCCGACCGATGTACACATTCTTGAGTG  90
BITS2-3 ..................................................................................................  90
BITS2-4 ..................................................................................................  90
CITS2-2 ..................................................................................................  90
BITS2-2 ..................................................................................................  90
CITS2-1 ..................................................................................................  90
UITS2-1 ------....-...N..........N..........N.........................N.....  64
UITS2-2 ------........N.....................................................N.....  65

            96          109                             141
            ↓           ↓                               ↓
BITS2-1 CCTACCAAATCCTTGTTACACAAATACATAACTACAGGACGGGCGTGCTACACAGTCAAGTGAAACATTCCGGGCGAGCAGCCCGCCACG 180
BITS2-3 .................................................................................................. 180
BITS2-4 .................................................................................................. 180
CITS2-2 .................................................................................................. 180
BITS2-2 .................................................................................................. 180
CITS2-1 .................................................................................................. 180
UITS2-1 .................................................................................................. 154
UITS2-2 .................................................................................................. 155

                                                    234     241
                                                    ↓       ↓
BITS2-1 GAAGTCACTGGACGTACACGCTGTCGGACTGTGCATCTTGGCGTGCTTGGTTCCACACCCAGAGCGGGGTGAACCTCGGGCGCTGAAAAG 270
BITS2-3 .................................................................................................. 270
BITS2-4 ..............................................................................A..... 270
CITS2-2 .................................................................................................. 270
BITS2-2 .................................................................................................. 270
CITS2-1 .................................................................................................. 270
UITS2-1 .................................................................................................. 244
UITS2-2 .................................................................................................. 245

            278
            ↓ **
BITS2-1 GTAAGGCAGAGTACAATCTTGTACACC-AGGGTACAGCGTCAAGTCGCACGGGTCGAACTTCGGCTATGGACGACCTGAGAACCCCGGCA 359
BITS2-3 ..........................-.....................................................T..... 359
BITS2-4 ..........--..............-..................................................... 357
CITS2-2 ..........--..............C..................................................... 358
BITS2-2 ..........................-..................................................... 359
CITS2-1 ..........................-..................................................... 359
UITS2-1 ..............N...........-..................................................... 333
UITS2-2 ...........N.NNN.N.N.N.NN..-..................................................... 334

                                                403                             434
                                                ↓                               ↓
BITS2-1 GCCTACTAACACCAGGCTAGCCGATCCGGGTCCAGGGGTTACGCATCATCCGGCCGAGTCGTGTAACACGTGCAACGCATACGCATGGAC 449
BITS2-3 .................................................................................................. 449
BITS2-4 ..............................................................T...C.G..... 447
CITS2-2 .................................................................................................. 448
BITS2-2 ..............................................................T..... 449
CITS2-1 ..............................................................C..... 449
UITS2-1 ..............................................................C..... 423
UITS2-2 ..............................................................S..... 424

            458             473
            ↓               ↓
BITS2-1 CCTACTTGCTTTCCTTTCGCATATGTAGGCCTCAAGTGATGTGTGACAACCCCCTGAATTTAAGCATA 517
BITS2-3 ..........................................                           517
BITS2-4 ..........................................                           515
CITS2-2 ..........................................                           516
BITS2-2 ..........................................                           517
CITS2-1 ..........................................                           517
UITS2-1 ..........................................-------                    464
UITS2-2 .....................................--------                        464
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Fig 3–Alignment of the ITS2 and flanking 5.8S and 28S regions of rDNA of *Anopheles aconitus* Form B, Form C and an unidentified form (data from GenBank). Dots indicate sequence identity with BITS2-1; dashes represent gaps introduced to maximize overall sequence similarity. The target sequences for PCR primers that flank the region analyzed are double-underlined. Arrows at 96 and 473 delineate the ITS2. Other arrows indicate positions of repeat elements described in the text. Asterisks (*) indicate positions of nucleotide deletion. Sequence names are defined in Table 2.

CTTTC at position 458 were also noted. Within 9 isolines of *An. aconitus* Form B, 4 different sequences (BITS2-1, BITS2-2, BITS2-3 and BITS2-4) were detected. Variation in the length of the isolines of Form B was present due to base deletion at positions 280 (A) and 281 (G) in BITS2-4. For Form C, 2 different sequences (CITS2-1, and CITS2-2) were obtained from 3 isolines. CITS2-2 was 1 nucleotide shorter than CITS2-1 as 2 bases were deleted at position 280 (A) and 281(G), and 1 base was inserted at position 298 (C). It is interesting to note that at position 436, Form B isolines showed mixed G/C/T, whereas Form C isolines showed mixed G/C. Sequence variation between the two forms was very low, ranging from 0.3 to 0.8% (1 to 3 base substitutions including sites of insertion and deletion).

Sequence analysis of the D3 region

Two different D3 sequences were obtained from 5 isolines of *An. aconitus* Form B (BD3-1 and BD3-2) and one D3 sequence from 4 isolines of Form C (CD3). Sequence alignment of the D3 region is shown in Fig 4. All D3 sequences of *An. aconitus* were 312 bp long, located between positions 43 and 354. BD3-1 and CD3 sequences were 100% identical. Compared to BD3-2, one nucleotide substitution at position 72 (C) was detected (variation rate = 0.3%). This position was a common site for base substitution in all the sequences compared.

Sequence analysis of COII

Fig 5 shows an alignment of COII and the flank region $tRNA^{Leu}$ and $tRNA^{Lys}$ of *An. aconitus* Form B and Form C. The COII genes of these

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                                          43                                  72
                                          ↓                                   ↓
BD3-2 GACCCGTCTTGAAACACGGACCAAGAAGTCTATCTTGCGCGCAAGCCAATGGGTATTGGGCGGTACGCCGCCTCGAACTGGTAACCCACA  90
UD3-1                      --------.....................................Y.....................  62
BD3-1                      .........................................................T.....  90
CD3                        .........................................................T.....  90
UD3-2                      -------.....................................YT.M.................  63

BD3-2 GGCGAAGACAAATCGAGTGTTGCGGGATTACGGGTTCGGCCGATGGCGCAAGCCTTCGTCGGGCCCCTCCATCCCAGGGTGTTCCGTTAC 180
UD3-1 ..................................................................................... 152
BD3-1 ..................................................................................... 180
CD3   ..................................................................................... 180
UD3-2 ..................................................................................... 153

BD3-2 GGGTGCTTGCACCCAGTGGACATCCCCGGAGTGCGTATGATGTGACCCGAAAGATGGTGAACTATGCCTGATCAGGTCGAAGTCAGGGGA 270
UD3-1 ..................................................................................... 242
BD3-1 ..................................................................................... 270
CD3   ..................................................................................... 270
UD3-2 ..................................................................................... 243

                                                                                  354
                                                                                  ↓
BD3-2 AACCCTGATGGAGGACCGAAGCAATTCTGACGTGCAAATCGATTGTCAGAGTTGGGCATAGGGGCGAAAGACCAATCGAACCATCTAGTA 360
UD3-1 .....................................................................................-      326
BD3-1 ..................................................................................... 360
CD3   ..................................................................................... 360
UD3-2 .....................................................................................--------- 319

BD3-2 GCTGGTTCCTTCCGA 375
UD3-1
BD3-1                 375
CD3                   375
UD3-2
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Fig 4–Alignment of the D3 region of 28S rDNA of *Anopheles aconitus* Form B, Form C and an unidentified form (data from GenBank). Dots indicate sequence identity with BD3; dashes represent gaps introduced to maximize overall sequence similarity. The target sequences for PCR primers that flank the region analyzed are double-underlined. Arrows at 43 and 354 delineate the D3 region. Sequence names are defined in Table 2. Y = C and T; M = A and C.

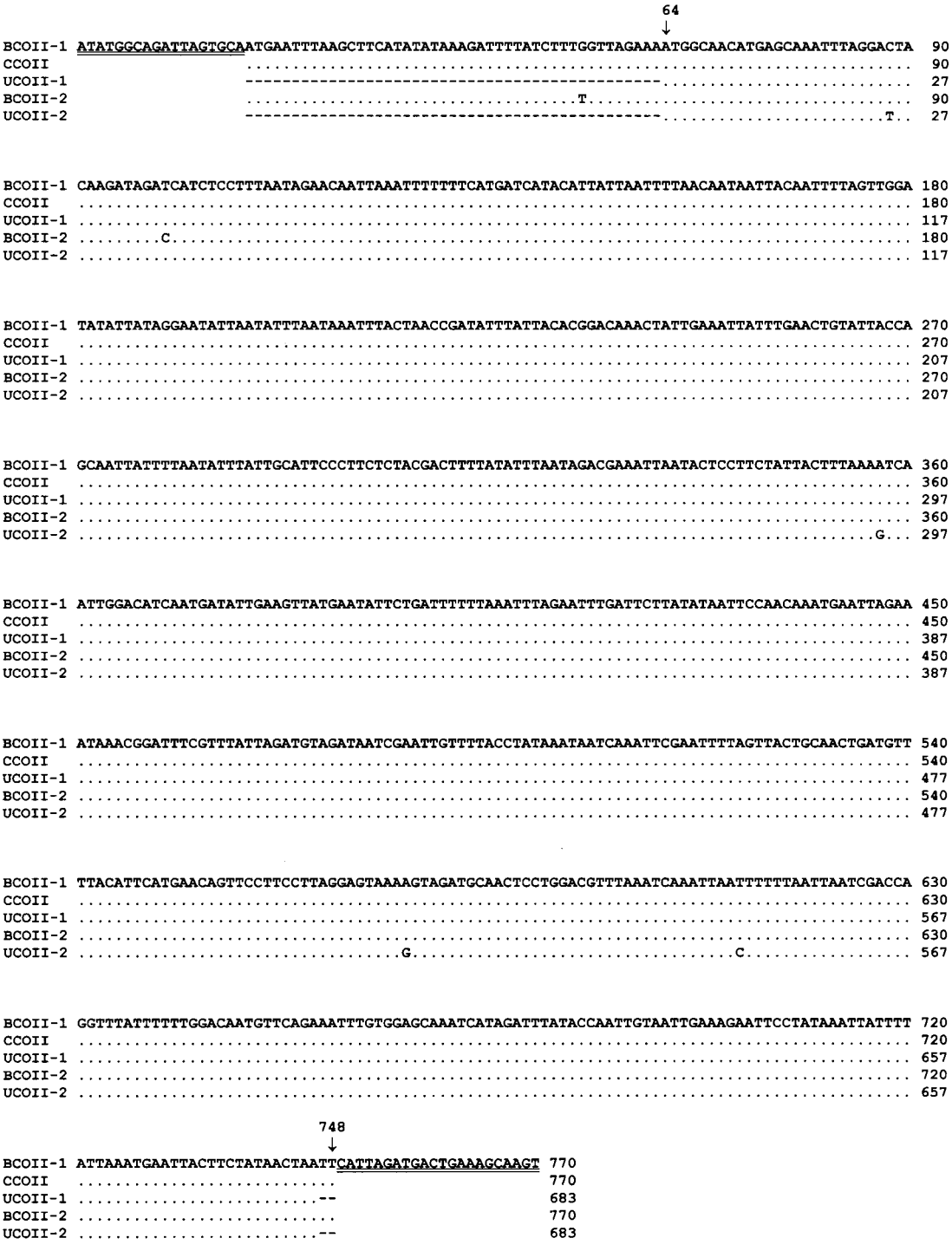

Fig 5–Alignment of the COII region of mtDNA of *Anopheles aconitus* Form B, Form C and an unidentified form (data from GenBank). Dots indicate sequence identity with BCOII-1; dashes represent gaps introduced to maximize overall sequence similarity. The target sequences for PCR primers that flank the region analyzed are double-underlined. Arrows at 64 and 748 delineate the COII sequence. Sequence names are defined in Table 2.

mosquito species were 685 bp in length. They had an ATG codon for initiation (A at position 64) and only the T at position 748 that potentially encodes the entire terminator. Two different COII sequences were obtained from 4 isolines of *An. aconitus* Form B (BCOII-1 and BCOII-2) and one COII sequence from 4 isolines of Form C (COII). Three COII sequences, BCOII-1, COII and UCOII-1, were the same. However, BCOII-2 and UCOII-2 showed 0.15% (1 base substitution) and 0.6% (4 base substitution) variation, respectively when compared with BCOII-1.

DISCUSSION

An. aconitus is one of the six members of the *An. minimus* group of *Anopheles* subgenus *Cellia* in the Myzomyia Series (Harrison, 1980). Based on metaphase karyotype studies, at least three karyotypic forms of *An. aconitus* (Forms A, B and C) have been reported sympatrically from Mae Taeng district, Chiang Mai Province, northern Thailand, whereas Form D (X_3, X_4, Y_4) has been incriminated in Java, Indonesia only (Baimai *et al*, 1996). Over the period of this study, no *An. aconitus* Form A was found. One possible explanation is that the Y_1 chromosome of *An. aconitus* consists of a small submetacentric figure, thus having a low amount of constitutive heterochromatin, and may have been lost from the population. The heterochromatin on eukaryotic chromosomes has a significant role in the regulation and concerted evolution of the genome. This may serve similar functions in the *An. aconitus* chromosomes. Therefore Form A might have become lost from the population, as Y_2 and Y_3 have extra block(s) of heterochromatin on the short and long arms of their chromosomes making males of Form B and Form C dominant in the population.

Previously intra- and inter-specific molecular variations have been investigated in four members of the Minimus group of *Anopheles* subgenus *Cellia*: *An. aconitus*, *An. varuna*, *An. minimus* A and C. DNA sequence divergence between these species at a mtDNA locus (COII) and at three nuclear loci (ITS2 and the D3 regions of rDNA and guanylate cyclase) has been reported (Sharpe *et al*, 2000). However, the DNA sequence data for *An. aconitus* is limited; only 24 sequences have been previously submitted to the GenBank database. These were derived from an unidentified form of *An. aconitus*. In this study, we isolated and sequenced the ITS1, ITS2 and D3 regions of the rDNA and COII genes for *An. aconitus* Form B and Form C. The consensus sequences of all loci were identical to those determined by Sharpe *et al* (2000). Sequence comparison of these four loci within and between the two forms showed great similarity with variation rates = 0.15 to 0.8%. These results correspond to those obtained in attempts to distinguish cryptic taxa in *An. gambiae s.s.* in West Africa. Four chromosomal forms of *An. gambiae s.s.* from West Africa were reported and DNA sequence variation in the ITS of rDNA, mtDNA and five unlinked single-copy nuclear loci were examined for evidence of reproductive isolation (della Torre *et al*, 2001; Favia *et al*, 2001; Gentile *et al*, 2001; Mukabayire *et al*, 2001). Although three sites in the ITS region distinguish the Mopti chromosomal form for Savanna and Bamako in Mali and Burkina Faso, outside these two countries the association between chromosomal form and DNA type does not always hold. In addition, two sequence-tagged random amplified polymorphic DNA (RAPD) loci, R15 and R37, have been reported as discriminating between Mopti and other chromosomal forms. However, neither loci has diagnostic value, and are not recommended as tools in the recognition of field-collected *An. gambiae* chromosomal forms. Their data suggest that gene flow among populations of this species is restricted and the molecular markers may not have the sensitivity required to detect recently established taxa of *An. gambiae s.s.*

The complete ITS1 sequence of *An. aconitus* is reported for the first time in this study. The region showed high variation (approximately 55%) compared to the closely related species *An. minimus* C. Detailed investigations of genetic divergences in this locus within the mosquitoes in the Minimus group remain to be undertaken.

The result of analysis of the ITS2 consensus sequence of *An. aconitus* is consistent with those of other studies that indicate a low frequency and variance of spacer mutants in the

genus *Anopheles*. Although no intraindividual variation was detected, intrapopulation variations were present with polymorphic sequences in some forms of each region, for example, ITS2-rDNA. Indels in regions of single-base repeats and simple repeat motifs account for most of the sequence variation observed and suggest their role as a major cause of divergence in the evolution of this spacer. In *An. aconitus*, sequence repeats, which may be subject to slipped-strand mis-pairing (SSM), are found at positions 280 and 281 of the ITS2 in the BITS2-4 and CITS2-2 sequences. Levinson and Gutman (1987) propose that the process of SSM is more likely to be a major factor in the initial expansion of short repeat motifs, which are subsequently predisposed to further expansion by unequal crossing-over. The rapid rate of fixation of such mutations in tandemly repeated genes may subsequently distinguish closely repeated species.

Williams *et al* (1987) analyzed the overall length and organization of the X- and Y-linked rDNA nontranscribed spacers of *Drosophila melanogaster* obtained from five continents and provided strong evidence that the X-linked rDNA arrays were under selective constraints. In *An. petragnani*, *An. hispaniola* (Marchi and Pili, 1994), *An. gambiae* (Kumar and Collins, 1994) and *An. stephensi* (Redfern, 1981), the rDNA genes were located on the sex pair, mainly within heterochromatic regions (C-banding) or adjacent to them. Recently, an rDNA (p*Dm* 238 – *D. melanogaster*) probe has been used to determine the relationship between the nucleolar organizer region (NOR) and constitutive heterochromatin (C-banding) in *An. darlingi* and *An. nuneztovari*. This probe mapped the X (X_1 and X_2) and Y chromosomes, whose gene sites coincided with the constitutive heterochromatin (C-banding) in the pericentromeric region and showed a conspicuous association with the NOR of both species. The gene sites agree with the data for X_1 acrocentric chromosomes of *An. darlingi* from Manaus and Macapa. In these chromosomes, the constitutive heterochromatin was located in the centromeric region, which extended to 1/3 of this chromosome, whereas the X_2 chromosomes showed fewer signals. In *An. nuneztovari* from Manaus, the intraspecific variations in the heterochromatic block signals in the submetacentric X_1 (longer) and the X_2 (shorter) chromosomes were the same as those of *An. nuneztovari* from Macapa (Rafael *et al*, 2003). In the case of *An. aconitus*, the location of rDNA in the chromosomes of each form should be examined, as the information could be useful for further study of the evolution of this species.

The gene for COII in the mtDNA has been frequently used in phylogenetic and population genetic studies. Sharpe *et al* (2000) combined D3 and COII data in the reconstruction of phylogenetic relationships of the Minimus group, because a phylogeny based on a single locus tree may not be the correct hypothesis, particularly for closely related taxa with large effective population sizes. The COII data for *An. aconitus* Form B and Form C was nearly identical (only 1 base substitution). But *An. aconitus* COII from the slightly allopatric area (Kanchanaburi Province, Thailand) showed a 4 base substitution (0.6% variation). It will be interesting to examine the COII in *An. aconitus* Form D as the form is only found in Indonesia. Different consensus sequences might be found in populations that inhabit different ecological zones, are separated by major geographic barriers, or are quite distant from each other. When geographically distant populations are found to differ in sequence, then the divergence is minimal and limited to regions that are prone to high rates of mutation.

Further analyses of rDNA, mtDNA and other genetic markers, including RAPDs, microsatellites of *An. aconitus* Form B, Form C and Form D, should provide information important to molecular taxonomy, evolutionary systematics, population genetics, genetic mapping, and the investigation of defined phenotypes for this species. However, the present ITS1, ITS2, D3 and COII data suggest that *An. aconitus* Forms B and C in Chiang Mai, Thailand are conspecific.

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