RESEARCH NOTE

The partial mitochondrial sequence of the Old World stingless bee, *Tetragonula pagdeni*

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Introduction

The partial mitochondrial sequence of *Tetragonula pagdeni* was examined using long PCR technique and subsequently cloned, and sequenced using primer walking strategy. The objective of this study was to characterize *T. pagdeni* mitochondrial DNA sequence, focussing on gene order. The partial mitochondrial sequence of *T. pagdeni* obtained in this study represents approximately 69.2% of the *Meliponini* mtDNA. The protein-coding genes have a bias towards AT-rich codons. Codon usage and amino acid sequences do not deviate substantially from those reported for other stingless bees or honeybees. Similar to other *Meliponini* species (Arias *et al.* 2006), the mitochondrial genome of *T. pagdeni* lacks the COI–COII intergenic region.

In past, mitochondrial DNA was widely employed to resolve population structure, phylogeography and phylogenetic relationships at various taxonomic levels (Kmiec *et al.* 2006). Generally, animal mtDNA exhibits a high evolution rate compared to that of the nuclear DNA and very conserved gene order comprising two rRNA genes, 22 tRNA genes, 13 protein-coding genes, as well as a noncoding control region (D-loop) (Wolstenholme 1992). The mtDNA has been extensively used in honeybee, *Apis mellifera*, to investigate natural range origin, and genetic polymorphisms based on PCR-RFLP method (Smith and Brown 1990). For stingless bees, the mitochondrial genome of several *Meliponine* species has been determined through RFLP analysis (Francisco *et al.* 2001). Besides, the nearly complete mtDNA sequence has been recently determined in stingless bee, *Melipona bicolor* (Silvestre *et al.* 2008). However, the information of mtDNA sequences from *Tetragonula* species is very limited, only few mitochondrial genes such as *16S rRNA* gene reported in genetic variability study (Costa *et al.* 2003; Rasmussen and Cameron 2007; Thummajitsakul *et al.* 2011). *T. pagdeni* Schwarz is widely distributed in Indo-Malayan/Australasian and Neotropical regions (Michener 2000) and is one of the most common indigenous stingless bees in Thailand (Sakagami 1978).

Materials and methods

Sample and DNA extraction

Individuals of *T. pagdeni* collected from a single colony were preserved in 95% ethanol and stored at 4°C until required. Total genomic DNA was extracted from each entire bee using phenol–chloroform extraction and ethanol precipitation following Thummajitsakul *et al.* (2011).

PCR and sequencing

In our previous studies, the mtDNA regions for *cytb*, *COI*, *16S rRNA* and *ATP(6, 8) + COIII* genes of *T. pagdeni* were amplified by using primer pairs; cyt-b-F/R, COI-F/R, LR13107-F/LR12647-R and ATPS6-F/tRNA-ASP-R, respectively (table 1). On the basis of each sequence portion, three sets of primer pairs LR12647-R + COI2494, LR12677 + cytb10729 and COIII9821 + cytb5031 were

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Table 1. Sequences of primers used in PCR reactions of *T. pagdeni* mtDNA.

mtDNA genes	Primer	Sequence
ATPase (6,8)	ATPS6-F	5′-AAG ATA TAT GGA AAT AAG CT-3′
	tRNA-ASP-R	5′-ATA AAA TAA CGT CAA AAT GTC A-3′
COI	COI-F	5′- ATA ATT ATT GTT GCT GAT GTA-3′
	COI-R	5′-CTA TTC ATA TAA CTG GAA TTT C-3′
Cytb	cyt b-F	5′-TTC ACT ATA TTA TAA AAG ATG TAA GTT C-3′
	cyt b-R	5′-GGC AAA AAG AAA ATA TCA TTC AGG-3′
16S rRNA	LR13107-F	5′-TGG CTG CAG TAT AAC TGA CTG TAC AAA GG-3′
	LR12647-R	5′-GAA ACC AAT CTG ACT TAC GTC GAT TTG A-3′
COI, COII, ATPase8, ATPase6, COIII, ND3, 12S, 16S rRNA	LR12647-R	5′-GAAACCAATCTGACTTACGTCGATTTGA-3′
	COI2494	5′-CGAGCATATTTTACATCAGCAACAAT-3′
Cytb, ND6, ND5, ND4, ND4L	LR12677	5′-GTTCAAATCGACGTAAGTCAGATTGGTTTC-3′
	cytb10729	5′-AGCTCCTCAAAATGATATTTGTCCTCATGG-3′
COIII, ND3, 12S, 16S rRNA, ND1, cytb	COIII9821	5′-TTAACGATAGAGTTTACGGGTCAAT-3′
	cytb5031	5′-AGCTACAGCATTTCTTGGGTATGTAC-3′

designed on each gene for the 16S/COI, 16S/cytb and cytb/COIII regions, respectively (table 1).

PCR was carried out with 30 cycles of a 25 μL reaction volume containing 4.75 μL of sterile distilled H_2O, 2.5 μL of 10× LA PCR buffer II (Takara Bio, Shiga, Japan), 4.0 μL of dNTP (2.5 mM), 5.0 μL of each primer (0.4 μM), 0.25 μL of 1.25 unit Takara LA Taq^{TM} (Takara), and 1.0 μL of template containing approximately 5 ng DNA. The PCR condition was used with denaturation at 98°C for 10 s and annealing and extension combined at the same temperature (60°C) for 10 min. The PCR products were verified on a 1.0% agarose gel and visualized by ethidium bromide staining via ultraviolet transillumination. Each purified PCR product was cloned into pGEM®-T easy vectors (Promega, Madison, USA) and subsequently sequenced by primer walking using BigDye terminator cycling conditions on a 3730xl automatic sequencer (Sequencing Service, Macrogen, Seoul, Korea). All sequences were analysed and compared by the homology search to assure the correct fragments using BLASTN (nucleotide similarity) available at http://www.ncbi.nlm.nih.gov.

Sequence analysis

Each coding region was identified by searching for open reading frames, including start and stop codons. The comparisons of nucleotide or amino acid sequences of *T. pagdeni* were performed by alignment with those of *M. bicolor* and *A. mellifera* using the BLASTX algorithm (NCBI). Transfer RNA and ribosomal RNA sequences were identified by eye comparison with homologues of *A. mellifera*, *M. bicolor* and *Bombus ignites*. Phylogenetic analysis using cyt-b mtDNA sequence among these bees was shown by neighbour-joining method in MEGA ver. 5.10 (Tamura *et al.* 2011) (figure 1).

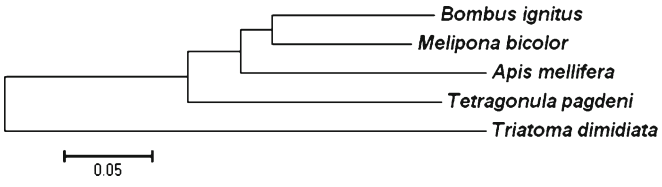

Figure 1. Phylogenetic analysis of cyt-b mtDNA genes among the bees was conducted using neighbour-joining method, using *Triatoma dimidiata* as outgroup (AF301594).

Results and discussion

The total size of stingless bees mtDNA has been estimated in *M. bicolor* species by RFLP analysis and sequencing as approximately 18,500 bp and 14,422 bp (Silvestre *et al.* 2008), respectively. In our study, the partial mtDNA sequence of *T. pagdeni* obtained was 12,802 bp or 69.2% of the total mtDNA genome of *Meliponini* species. The incomplete mtDNA sequence contained 11 complete genes, a partial COI gene, both rRNA genes and 12 tRNA genes. Each protein-coding gene in *T. pagdeni* was similar in length and nucleotides to their counterpart genes in other stingless bees (*M. bicolor*) and honey bees (*A. melliferra*) (table 2). The four overlapping regions, *ND4/ND5*, *cytb/tRNA-Ser* (*S2*), *ND1/tRNA-Leu* (*L1*) and *ATP8/ATP6*, were observed (figure 2). Some of the overlapped genes have been reported for *M. bicolor* mtDNA by Silvestre *et al.* (2008) such as *ND1* and *tRNA-Leu* (*L1*) shared six nucleotides; *ATP6* and *ATP8* shared 10 nucleotides. Moreover, 14 noncoding regions were observed with total intergenic region of 419 bp. In honeybee *A. mellifera*, the number of noncoding nucleotides, excluding the *COI–COII* intergenic and control region is 618 bp (Crozier and Crozier 1993).

The noncoding region found in mtDNA sequences of *T. pagdeni* showed no significant similarities with any regions of the mtDNA of *M. bicolor*. The intergenic region between *COI* and *COII* genes of *A. mellifera* mtDNA is known as hypervariable region. This intergenic region has been widely studied in *A. mellifera*, and size polymorphism

Table 2. Size, start codon and stop codon comparisons of protein-coding genes between *T. pagdeni* (Tp), *M. bicolor* (Mb) and *A. mellifera* (Am).

	Length (bp)			Start codon			Stop codon			% Nucleotide similarity between two species	
Gene	Tp	Mb	Am	Tp	Mb	Am	Tp	Mb	Am	Tp and Mb	Tp and Am
ND4L	300	279	264	ATA	ATA	ATT	TAA	TAA	TAA	69%	68%
ND4	1392	1323	1344	ATG	ATT	ATA	TAG	TAA	TAA	78%	71%
ND5	1665	1647	1665	ATC	ATT	ATT	TAA	TAA	TAA	76%	70%
ND6	522	540	504	ATT	ATT	ATT	TAA	TAA	TAA	71%	69%
cytb	1089	1050	1152	ATT	ATT	ATG	TAA	TAA	TAA	79%	74%
ND1	933	930	918	ATA	ATA	ATT	TAA	TAA	TAA	76%	72%
ND3	351	354	354	ATA	ATA	ATA	TAA	TAA	TAA	71%	0%
COIII	780	780	777	ATG	ATG	ATG	TAG	TAA	TAA	70%	67%
ATPase6	687	684	681	ATG	ATG	ATG	TAG	TAA	TAA	69%	66%
ATPase8	168	168	159	ATT	ATT	ATT	TAA	TAA	TAA	72%	73%
COII	627	678	676	ATT	ATT	ATT	TAG	TAA	T	76%	71%

has been reported (from 200 to 650 bp) among subspecies (Garnery *et al.* 1992, Franck *et al.* 1998). It has also been referred as a possible second origin of mtDNA replication and transcription (Cornuet *et al.* 1991). Our results reveal that the COI–COII intergenic region was absent in *T. pagdeni*, as has been previously reported in the case of *M. bicolor* and at least 16 other *Meliponini* species (Arias *et al.* 2006).

The A + T contents of each protein-coding gene in *T. pagdeni* were high, ranging from 66 to 87% (table 3), similar to *M. bicolor* (87%, Silvestre *et al.* 2008) and *A. mellifera* (85%, Crozier and Crozier 1993). The highly biased A + T content has been known as a major characteristic of the mitochondrial genome of *M. bicolor* and *A. mellifera* (Crozier and Crozier 1993; Silvestre *et al.* 2008). The advantage of AT bias could be explained by one hypothesis that the DNA polymerase could use those bases in a more efficient way during mtDNA replication (Clary and Wolstenholme 1985) because the energetic cost to break A–T links is lower compared to G–C links. The A + T-rich region of *A. melifera* is located between the *12S rRNA* and *tRNA-Glu* genes (Crozier and Crozier 1993). It contains several short repeating sequences (6–13 bp) with varying copy numbers (two-to-four copies), scattered through the whole region and a polythymidine stretch. This polythymidine stretch is reported to be a transcription control or the initiation of replication (Zhang and Hewitt 1997). For stingless bee *M. bicolor*, the A + T-rich region could not be sequenced because of difficulties in amplification

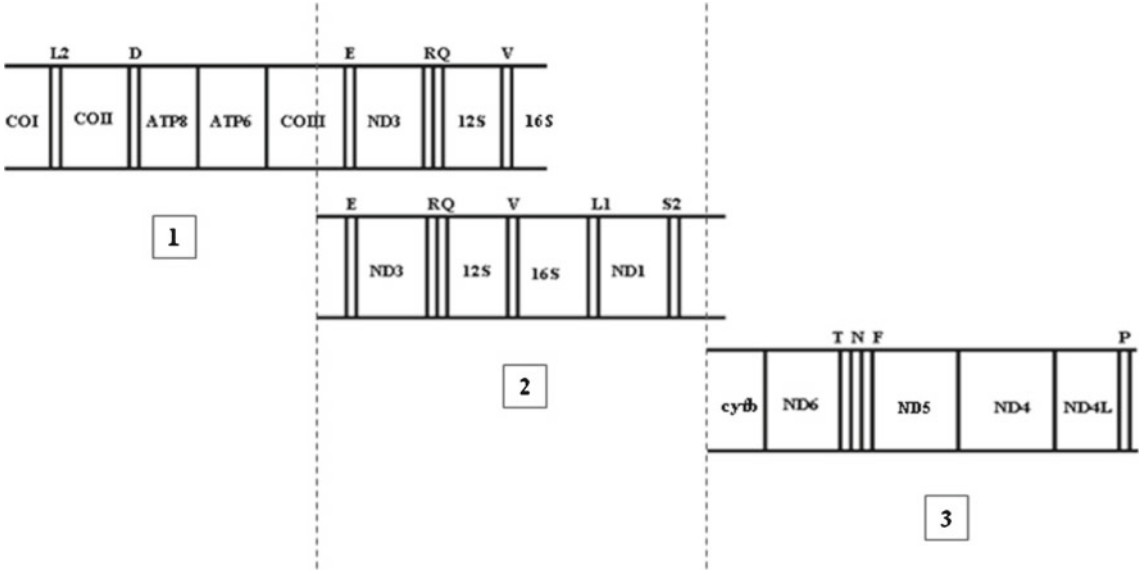

Figure 2. Overlapping the three sequenced fragments (5855, 4879 and 5089 bp, respectively) obtained from long PCR amplification with LR12647-R + COI2494, COIII9821 + cytb5031 and LR12677 + cytb10729, respectively and sequenced by primer walking.

Table 3. Presentation of base compositions (%) in each gene of *T. pagdeni* mtDNA.

Gene	Length	A + T content (%)
ND4L	300	87
ND4	1392	83
ND5	1665	83
ND6	522	86
cytb	1089	77
ND1	933	83
ND3	351	66
COIII	780	68
ATPase6	687	66
ATPase8	168	80
COII	627	69
16S rRNA	1351	77
12S rRNA	762	76
tRNA-Pro	67	84
tRNA-Phe	67	85
tRNA-Asn	68	82
tRNA-Thr	67	79
tRNA-Ser(2)	67	85
tRNA-Leu(1)	69	83
tRNA-Val	68	88
tRNA-Gln	68	79
tRNA-Arg	61	85
tRNA-Glu	65	71
tRNA-Asp	81	78
tRNA-Leu(2)	65	75

(Silvestre *et al.* 2008), but it was described by its size using RFLP analysis. It was estimated in size approximately 3300 bp, about 2.5-kb longer than in *A. mellifera* (Crozier and Crozier 1993). In this study, because we were not able to amplify the entire mtDNA of *T. pagdeni*, the A + T-rich region, ND2 and 10 tRNA genes were not found on the sequenced mtDNA fragment. The DNA amplification failure can be explained by the existence of tandem repeats, heteroplasmy and great length variation at intraspecific and interspecific levels (Zhang and Hewitt 1997), including partial duplications that commonly occur in insect mtDNA. All protein-coding genes of *T. pagdeni* indicated slight differences when compared to *M. bicolor* and *A. mellifera*. The nucleotide sequence of *T. pagdeni* is more similar to *M. bicolor* than *A. mellifera*.

The 12S and 16S rRNA genes of *T. pagdeni* mtDNA were estimated to be 762 and 1351 bp, respectively, and showed 83% and 78% similarity compared to the partial 12S (437 bp) complete 16S rRNA (1354 bp) nucleotide sequence genes of *M. bicolor*, respectively. The srRNA and lrRNA genes of *T. pagdeni* were located between the tRNA-Leu (1) and tRNA-Val gene and between the tRNA-Val and tRNA-Gln genes, respectively (figure 3). The order of *12S rRNA*, *16S rRNA*, *ND1*, *cytb* and *ND6* genes of *T. pagdeni* mtDNA was different from those of *M. bicolor* (Silvestre *et al.* 2008). All tRNA sequences had 61–81 nucleotides with 12 to 29% G + C and 70.77 to 88% A + T (data not shown). The anticodon nucleotides were identical to those commonly found for the corresponding tRNA genes in *A. mellifera*, *M. bicolor* and *B. ignitus* mtDNA. However, tRNA-Gln found in *T. pagdeni* mtDNA resembled those in *A. mellifera* and *B. ignitus* mtDNA, but not seen in *M. bicolor* mtDNA (Crozier and Crozier 1993; Silvestre *et al.* 2008). The tRNA-Gln gene was located between 12S and tRNA-Arg (figure 2).

In conclusion, the nearly complete mitochondrial genome of *T. pagdeni* may provide critical information for future mtDNA analyses on biology, ecology and evolution at intraspecific and interspecific levels of other *Tetragonula* species. Studies of mtDNA gene organization illustrate an expedited rate in hymenopterans (Dowton *et al.* 2002). The mitochondrial gene order could be explained by several mechanisms. One of the most extensively-accepted

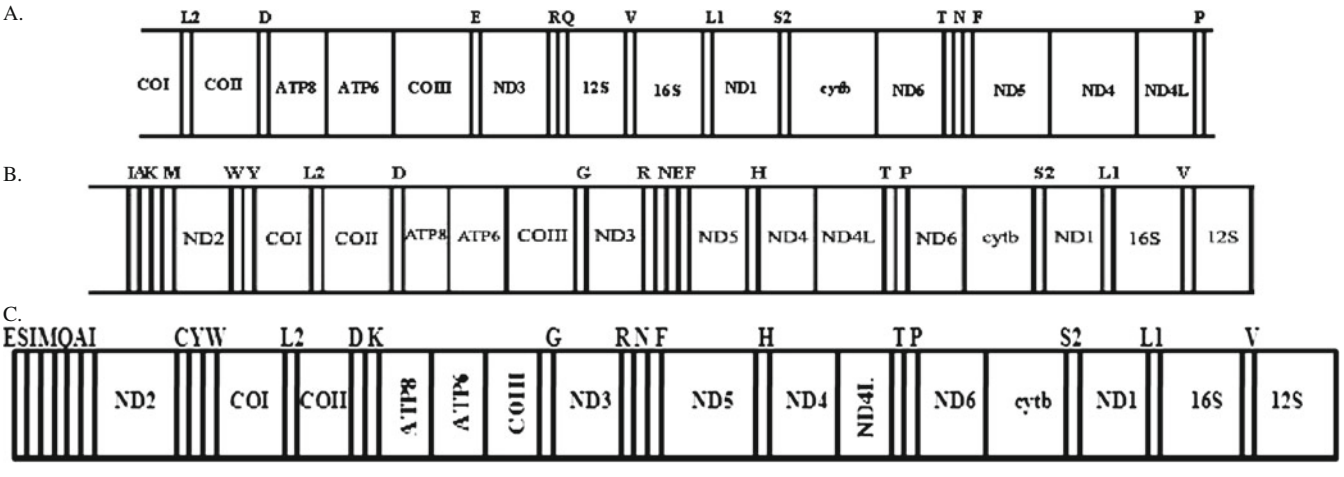

Figure 3. Gene organization of the mtDNA from *T. pagdeni* (A), *M. bicolor* (B) (Silvestre *et al.* 2008) and *A. melliferra* (Crozier and Crozier 1993). All *tRNA* genes were indicated by the amino acid they encode: I (tRNAIle), A (tRNAAla), K (tRNALys), M (tRNAMet), W (tRNATrp), Y (tRNATyr), L2 (tRNALeu(UUR)), D (tRNAAsp), G (tRNAGly), R (tRNAArg), N (tRNAAsn), E (tRNAGlu), F (tRNAPhe), H (tRNAHis), T (tRNAThr), P (tRNAPro), S2 (tRNASer(UCN)), L1 (tRNALeu(CUN)) and V (tRNAVal).

mechanisms is tandem duplication of gene regions as a result of slipped-strand mispairing followed by gene deletions (Levinson and Gutman 1987; Macey *et al.* 1998).

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