CHAPTER II

DEVELOPMENT OF SPECIES-DIAGNOSTIC MARKERS FOR IDENTIFICATION OF THE STINGLESS BEE (*Trigona pagdeni* Schwarz) IN THAILAND

INTRODUCTION

The eusocial stingless bees (Apidae, Apinae, Meliponini) are widely distributed over tropical and subtropical regions worldwide (Michener, 1974; 1990; 2000). They are naturally effective crop pollinators (Amano *et al.*, 1999; Heard, 1999; Slaa *et al.*, 2000), and many species have been used in traditional beekeeping (Crane, 1992). A total of 32 stingless bees, which are allocated into a genus *Trigona*, are known in Thailand (Klakasikorn *et al.*, 2005; Michener and Boongird, 2004). Of these, *T. pagdeni* is predominant and commonly found covering vast geographic locations in Thailand, peninsular Malaysia and the Indochina region (Sakagami, 1978; Sakagami and Khoo, 1987).

Generally, the stingless bees have been taxonomically classified based principally on morphology and nest architecture characters (Michener, 1961; Sakagami, 1978; Dollin *et al.*, 1997). Nevertheless, taxonomic identification of stingless bees remains unclear and requires experienced scientists, especially the taxonomic difficulties of the subgenus *Tetragonula* of *Trigona* Jure 1807 (Schwarz, 1939; Baltazar, 1966) which were abundantly found in Southeast Asia (Sakagami, 1978). The external characteristics are influenced by a variety of habitats and environmental conditions. Nest architecture characters are usually relevant but were reported that they were not sufficient criteria for authentication of species origin of Australian stingless bees (e.g. *T. hockingsi* and *T. davenporti*; Franck *et al.*, 2004). Many *Trigona* are sympatric species (e.g. between *T. pagdeni* and *T. fuscobalteata*; Sakagami, 1978) and could not be preliminary distinguished based on geographic distribution. In addition, species recognition of stingless bees is more complicated by the existence of cryptic species (e.g. between *T. carbonaria* and *T. hockingsi* and between *T. iridipennis* and *T. laeviceps*; Starr and Sakagami, 1987). Systemetics of stingless bee can be carried out using DNA based technology. Species-diagnostic

markers are thus required to unambiguously identify species origins of there taxa for population genetic studies of *T. pagdeni*.

The objective of this study is to develop reliable species-diagnostic markers to distinguish *T. pagdeni* from other stingless bees in Thailand. An AFLP fragment specifically found in *T. pagdeni* was cloned and characterized. A potential *T. pagdeni* -diagnostic AFLP-derived marker was developed and further analyzed by SSCP.

MATERIALS AND METHODS

Sampling

Adult *Trigona* workers of each colony were collected from different localities in Thailand (Figure A.1 and Table A.1, APPENDIX A). Specimens were placed in 95% ethanol and kept at 4°C until required. Taxonomic identification of the stingless bees samples was carried out based on nest architecture and morphology according to Sakagami (1978) and Sakagami and coworkers (1983), and later confirmed by Professor Dr. Charles D. Michener (University of Kansas).

DNA extraction

Total DNA was extracted from each stingless bees using a phenol-chloroform-SDS method (Smith and Hagen, 1996). DNA concentration was estimated by comparing with that of undigested lambda DNA using a minigel method (Sambrook and Russell, 2001).

AFLP analysis

AFLP analysis was carried out as essentially described by Vos and coworkers (1995). Briefly, pooled genomic DNA (250 ng) of bees from the same colony was digested with *Pst*I and *Mse*I prior to ligate with restriction site-specific adaptors. Preamplification was carried out utilizing adaptor specific primers with a single selective base on each primer; *Pst*I primer (5'-GACTGCGTACATGCAG**A**-3') and *Mse*I primer (5'-GATGAGTCCTGAGTAA**C**-3'). The preamplification product was diluted 25-fold, and selectively amplified using primers with an additional 2 selective bases at the 3' terminus of each primer (64 primer combinations; Table A.2;

APPENDIX A). AFLP fragments were size-fractionated through 4.5% polyacrylamide sequencing gels and visualized by silver staining.

Cloning and sequencing of candidate species-specific AFLP fragment

A 284 bp fragment found in *T. pagdeni* but not in other stingless bees was excised, eluted out from the gel and reamplified. The product was cloned into pGEM®-T Easy vector (Hoelzel and Green, 1992). The insert sizes were verified by colony PCR. Plasmid DNA was extracted from recombinant clones and sequenced for both directions.

PCR-SSCP analysis

A pair of primers (CUTPTP1-F; 5'-CATCTGGTTGTCGGTCTGTAA-3' and CUTPTP1-R; 5'-TTCCTTCTCCTAATCTTTGCGG-3') was designed from the characterized AFLP fragment and tested across 15 *Trigona* species (Table 2.1). PCR amplification of the extracted DNA was carried out in a 25 µl reaction volume which usually contained 10 mM Tris-HCl (pH 8.8), 50 mM KCl and 0.1% Triton X-100, 2.0 mM $MgCl_2$, 100 µM dNTPs, 1 unit of DyNAzyme™ DNA polymerase (Finnzymes, Finland), 0.1 µM of each primer. PCR was performed involving predenaturation at 95°C for 3 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 50 seconds, and extension at 72°C for 30 seconds. The final extension was performed at 72°C for 7 minutes.

For SSCP analysis, 5 µl of the purified amplification product of each bee (a 163 bp CUTPTP fragment of *T. pagdeni* (n = 64), *T. fuscobalteata* (n = 15) ,*T. collina* (n = 11), *T. laeviceps*(n = 1), and *T. fimbriata* (n = 1)), was mixed with 4 volumes of the loading dye (95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol and 10 mM NaOH), denatured at 95°C for 3 minutes, immediately cooled on ice for 2 minutes and electrophoretically analyzed through 17.5% non-denaturing polyacrylamide gels (37.5:1 crosslink) at 12.5 V/cm for 16 hours at 4°C. The SSCP bands were visualized by silver staining.

Table 2.1 Sampling locations and sample sizes of stingless bees used in the species-specific test using CUTPTP primers

Location/Species	Tapi	Tcol	Tcan	Tfim	Tita	Tlae	Tfus	Tpag	Tmel	Tmin	Tdoi	Tmela	Tter	Ttho	Lfur
NORTH															
Chiang Mai	2	1	-	2	-	-	5	4	-	-	1	1	-	-	-
Nan	-	2	-	-	-	-	-	2	-	2	-	-	-	-	-
Phrae	-	2	-	-	-	5	-	-	-	-	-	-	-	-	-
Sukothai	-	-	-	-	-	-	-	5	-	-	-	-	-	-	-
Uttaradit	-	-	-	-	-	-	1	-	-	5	-	-	1	-	-
Uthaithani	-	5	-	-	-	-	-	-	-	-	-	-	-	-	-
Kampaeng Phet	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-
Phisanulok	-	-	-	-	-	2	-	2	-	-	-	-	-	-	-
Phichit	-	4	-	-	-	5	-	-	-	-	-	-	-	-	-
NORTH-EAST															
Udorn Thani	-	2	-	-	-	-	-	1	-	-	-	-	-	1	-
Khon Kaen	-	5	-	-	-	-	-	3	-	-	-	-	-	-	-
Maha Sarakam	-	2	-	-	-	-	-	3	-	-	-	-	-	-	-
Roi Et	-	2	-	-	-	-	-	6	-	1	-	-	3	-	-
Sakon Nakorn	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-
Chaiyaphum	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-
Srisaket	-	3	-	1	-	-	-	1	-	4	-	-	-	-	-
Surin	-	1	-	-	-	-	-	4	-	-	-	-	-	-	1
Mukdahan	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
Ubon Ratchatani	-	4	-	-	-	-	-	4	-	2	-	-	3	-	-
Burirum	-	2	-	-	-	-	-	2	-	-	-	-	-	-	-
Nakhon Ratchasima	-	1	-	-	-	-	-	-	-	-	-	-	-	-	1
CENTRAL REGION															
Sing Buri	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-
Loburi	-	-	-	-	-	-	-	3	-	-	-	-	-	-	-
Ayutthaya	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-

Table 2.1 (continued)

Location/Species	Tapi	Tcol	Tcan	Tfim	Tita	Tlae	Tfus	Tpag	Tmel	Tmin	Tdoi	Tmela	Tter	Ttho	Lfur
Nonthaburi	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-
Suphan Buri	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-
Bangkok	-	-	-	-	-	-	-	4	-	-	-	-	-	-	-
Kanchanaburi	-	14	-	-	-	-	7	10	-	-	-	-	-	-	-
Ratchaburi	4	1	-	-	-	-	-	-	-	-	-	-	2	1	1
Petchaburi	-	-	-	-	-	-	-	4	-	-	-	-	-	-	-
Trat	-	-	-	-	-	-	2	1	-	-	-	-	-	-	-
Chanthaburi	-	-	-	-	-	-	-	3	-	-	-	-	-	-	-
Chon Buri	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-
Prachuap Khiri Khan	-	14	-	-	-	-	-	20	-	-	-	-	-	-	-
PENINSULAR THAILAND															
Chumphon	2	31	-	-	-	-	-	16	-	-	-	-	-	-	-
Ranong	-	5	-	-	-	-	-	1	-	-	-	-	-	-	-
Krabi	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-
Surat thani	-	2	1	-	2	-	-	6	1	-	-	-	-	1	-
Nakhon Si Thammarat	1	-	-	-	-	-	-	1	-	-	-	-	-	-	-
Songkhla	-	-	-	-	3	-	-	10	-	1	-	-	-	1	-
Phatthalung	-	-	-	-	-	-	-	4	-	-	-	-	-	-	-

Tapi = *T. apicalis*, Tcol = *T. collina*, Tcan = *T. canifrons*, Tfim = *T. fimbriata*, Tita = *T. itama*, Tlae = *T. laeviceps*, Tpag=*T. pagdeni*, Tfus= *T. fuscobalteata* Tmel = *T. melina*, Tmin = *T. minor*, Tdoi = *T. doipaensis*, Tmela= *T. melanoleuca*, Tter = *T. terminata*, Ttho = *T. thoracica*, Lfur = *Lisotrigona furva* Engel

RESULTS

Genomic DNA was isolated from each individual bee using phenol-chloroform extraction (Smith and Hagen, 1996). The DNA concentration was estimated by comparison of the intensity of DNA-ethidium bromide complex with a known quantity of digested lambda DNA on a mini-gel (Sambrook and Russell, 2001). The extracted DNA showed high molecular weigh at 23.1 kb with smear (Figure 2.1). The OD_{260}/OD_{280} ratio of the extracted DNA was approximately ranged from 1.8 to 2.0 indicating the extracted DNA was relative pure (Sambrook *et al.*, 2001). This extracted DNA was subjected to AFLP analysis.

AFLP analysis

The AFLP analysis consisted of two steps of PCR amplifications: preamplification step and selective amplification step. In the preamplification step, when primer combination (*Pst*I$_{+A}$ and *Mse*I$_{+C}$ primer) was used, the smear preamplification products with the molecular weight greater than 200 bp was observed (Figure 2.2). This indicated the successful of digestion and ligation of *T. pagdeni* genomic DNA. The preamplification products were then used as the template in the selective amplification. For the selective amplification step, 64 primer combinations (Table A.2, APPENDIX A) were screened to test against genomic DNA of 26 *T. pagdeni* and other 11 stingless bees species. The products of the selective amplification were analyzed by agarose gel electrophoresis. It showed different band patterns in each *Trigona* species using the same primer combination (Figure 2.3). It implied that successful development of the genomic DNA for further screening of species-specific markers in *T. pagdeni* by AFLP analysis was accomplished.

The primer combinations provided a low level of polymorphism in *T. pagdeni* and different band patterns from other *Trigona* species were screened to search for species-specific markers in *T. pagdeni*. The *Pst*I$_{+ATC}$ and *Mse*I$_{+CAG}$ primer combination was the best choice for this propose. Likewise, 11 bands ranged from 110 to 470 bp were scored in *T. pagdeni*, and a 284 bp fragment was solely found in *T. pagdeni* (Figure 2.4).

Cloning and characterization of a species-specific AFLP fragment

A 284 bp fragment found only in *T. pagdeni* was successfully reamplified (Figure 2.5). The purified PCR product was cloned. Colony PCR was performed to verify of the insert sizes (Figure 2.6). The recombinant plasmid DNA was used for DNA sequencing for both directions. BLAST*N* and BLAST*X* data search for homology sequences did not reveal significant similarity with any gene sequences previously deposited in the GenBank. Therefore, it was regarded as unknown sequences (Figure 2.7).

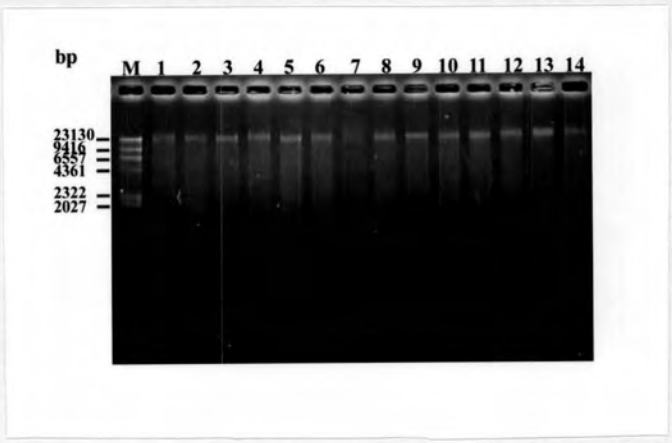

Figure 2.1 A 0.8% ethidium bromide stained agarose gel showing the quality of the extracted DNA of each bee. Lane M is 100 ng of λ–*Hin*dIII and Lanes 1-14 are genomic DNA extracted from each entire bee of *T. pagdeni*.

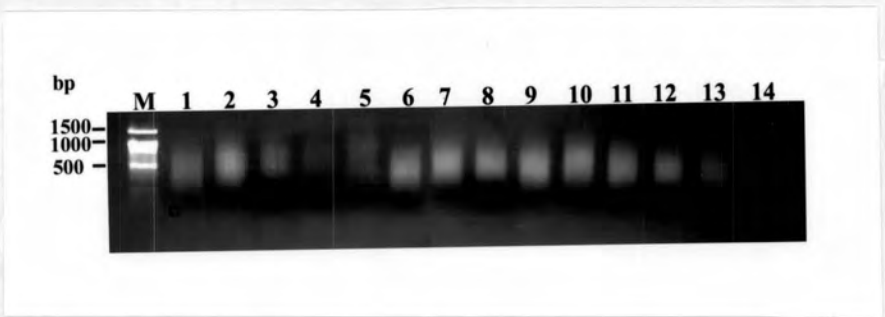

Figure 2.2 A 1.8% agarose gel electrophoresis of preamplification products of genomic DNA of each *Trigona* species; *T. pagdeni* (lanes 1 and 14), *T. collina* (lanes 2 - 4), *T. melina* (lane 5), *T. apicalis* (lanes 6 and 10), *T. itama* (lane 7), *T. thoracica* (lane 8), *T. canifrons* (lane 9), *T. melanoleuca* (lane 11), *T. fimbriata* (lane 12), *Lisotrigona furva* Engel (lane 13). Lane M is a 100 bp DNA marker.

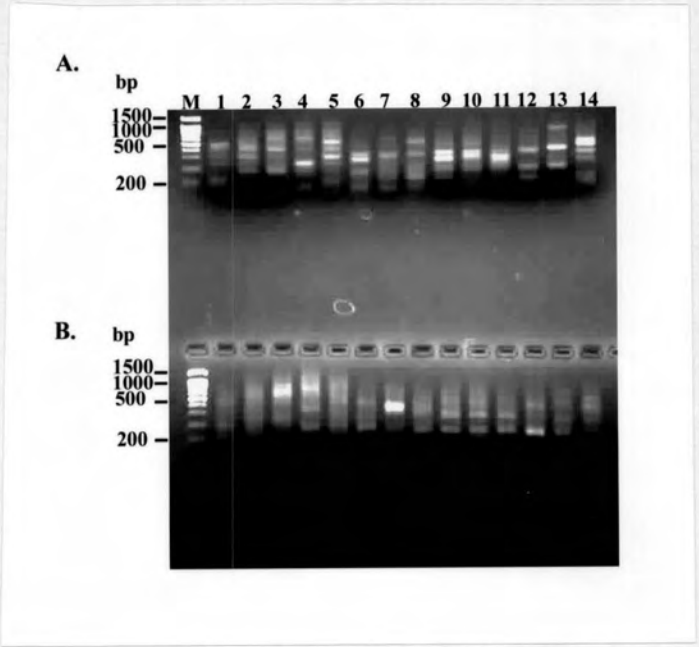

Figure 2.3 A 1.8% agarose gel electrophoresis of selective amplification products of genomic DNA of each *Trigona* species using two primer combinations; *Pst*I$_{+ATC}$/*Mse*I$_{+CAG}$ (A), *Pst*I$_{+ATG}$/*Mse*I$_{+CAG}$ (B); *T. pagdeni* (lanes 1 and 14), *T. collina* (lanes 2 - 4), *T. melina* (lane 5), *T. apicalis* (lanes 6 and 10), *T. itama* (lane 7), *T. thoracica* (lane 8), *T. canifrons* (lane 9), *T. melanoleuca* (lane 11), *T. fimbriata* (lane 12), *Lisotrigona furva* Engel (lane 13). Lane M is a 100 bp DNA marker.

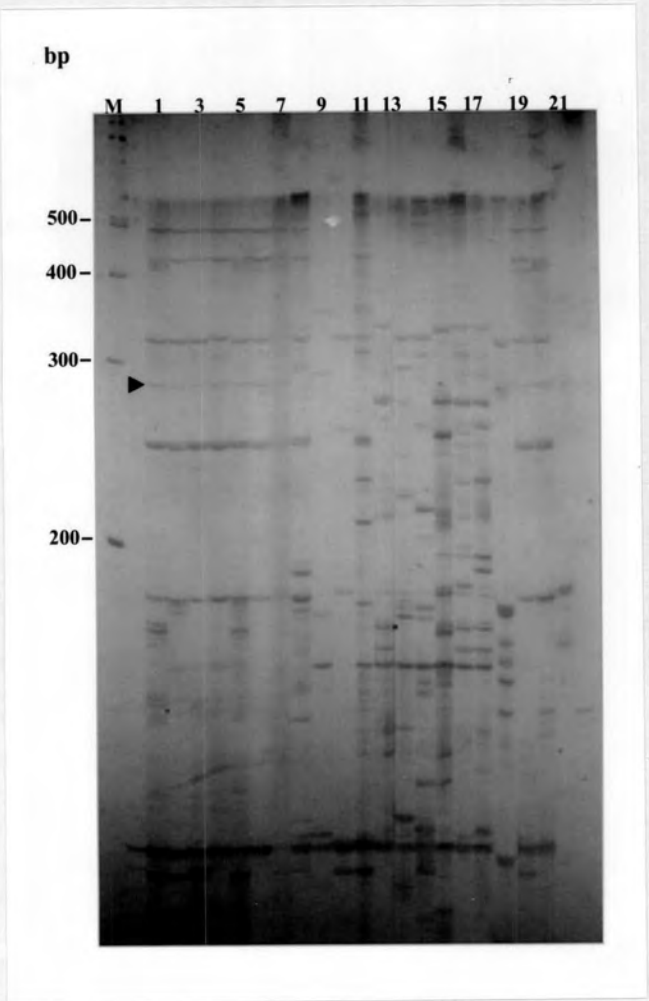

Figure 2.4 AFLP patterns of stingless bees; *T. pagdeni* (lanes 1 to 7, 19 and 20), *T. collina* (lane 8) , *T. terminata* (lane 9), *T. minor* (lane 10), *T. melina* (lane 11) , *T. apicalis* (lanes 12 and 16), *T. itama* (lane 13), *T. thoracica* (lane14) , *T. canifrons* (lane15), *T. melanoleuca* (lane 17), *T. fimbriata* (lane 18), *Lisotrigona furva* Engel (lane 21B) genotyped by *Pst*I$_{+ATC}$/*Mse*I$_{+CAG}$. Lanes M are a 100 bp DNA ladder. An arrowhead indicates a 284 bp band specifically found in *T. pagdeni*.

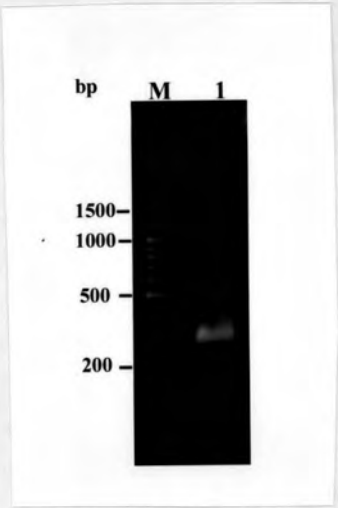

Figure 2.5 Reamplification of the species-specific marker of *T. pagdeni*. Lane M is a 100 bp ladder.

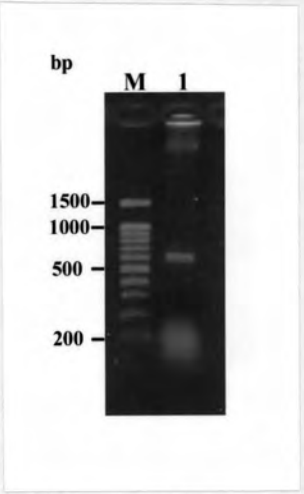

Figure 2.6 Colony PCR product of the recombinant clone containing the targeted insert (the species-specific AFLP marker in *T. pagdeni*). Lane M is a 100 bp ladder.

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5'GACTGCGTAC ATGCAGATCA CTCGAATCGG AAGAAAACAA ATGGAAACAT
  CTGGTTGTCG GTCTGTAACA CGGCAAAACC CTTGGAATAT TCCCGTTTCC
  CGAAAAATGT TCGAGACCGT GTCGAACGAT AACTAATCGA TACTCGAGCG
  CACGGCGGAG AGGAGAAAGG GGACGAGAAG GGAGGGAGAA AGGAAGAGGA
  TTAGAACGCC CTCTAGAAAT CAGTAAATCT AGAAACGATC TCTCGATGTT
  TCACATGTCA TTGACTGTTA CTCAGGACTC ATCA 3'
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Figure 2.7 Nucleotide sequence of a species-specific AFLP fragment (284 bp) in *T. pagdeni.* The locations and sequences of the species-specific forward primer (CUTPTP1-F) and those complementary to a reverse primer (CUTPTP1-R) are underlined.

Development of species-specific SCAR (sequence characterized amplified region) marker in *T. pagdeni*

A primer pair (CUTPTP1-F and CUTPTP1-R) was derived from the sequence of the species-specific AFLP fragment (Figure 2.7). Species-specificity of the SCAR marker was tested against genomic DNA of *T. pagdeni* and other stingless bees (Figure 2.8). The expected product (163 bp) was consistently found in *T. pagdeni* (129/129 accounting for 100% of investigated individuals) but not in *T. apicalis*, *T. canifrons*, *T. itama*, *T. melina*, *T. minor*, *T. terminata*, *T. doipaensis*, *T. melanoleuca* and *T. thoracica* and the outgroup *Lisotrigona furva* Engel. Nevertheless, cross-species amplification was found in *T. fimbriata* (1/3, 33.3% subgenus *Homotrigona*), *T. collina* (11/112, 9.8%; the atripes group in subgenus *Tretragonula*), *T. laeviceps* (1/12, 8.3%; the iridipennis group in subgenus *Tretragonula*) and *T. fuscobalteata* (15/15, 100%; the iridipennis group in subgenus *Tretragonula*). The SCAR marker was not solely found in *T. pagdeni*. It implied that conversion of the species-specific AFLP marker to species-specific SCAR marker was unsuccessful (Figure 2.8 and Table 2.2).

Characterization of the SCAR marker using SSCP analysis

The SCAR marker derived from a species-specific AFLP marker did not reveal species-specificity. Therefore, the SSCP analysis was performed to characterize the amplified fragments found in *T. pagdeni* (n = 62), *T. fimbriata* (n = 1) *T. collina* (n = 11), *T. laeviceps* (n = 1) and *T. fuscobalteata* (n = 15). The result of the SSCP analysis provided monomorphic banding patterns against investigated individuals of *T. pagdeni*, *T. laeviceps* and *T. fimbriata* and different banding patterns from *T. collina* and *T. fuscobalteata* (Figure 2. 9).

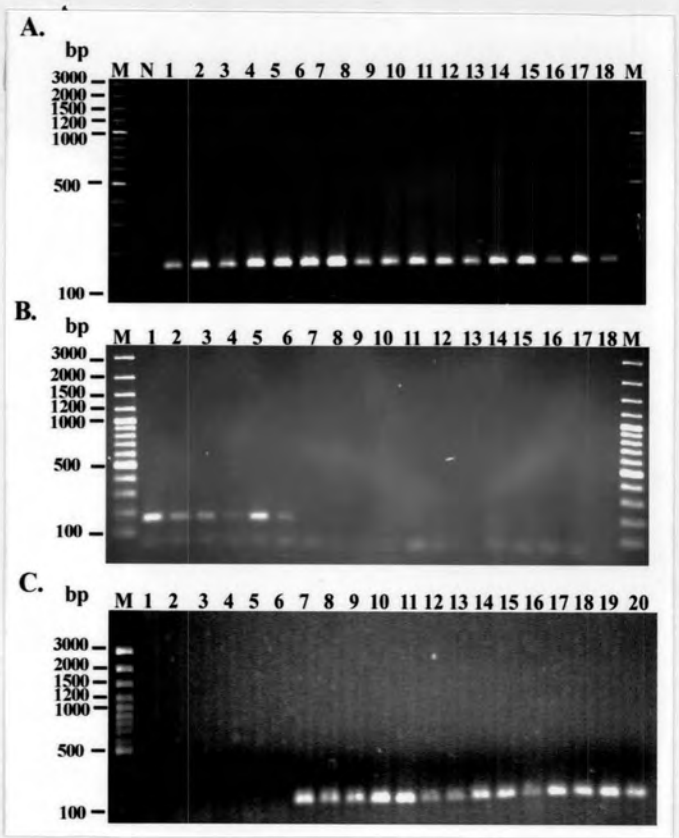

Figure 2.8 A 1.5% agarose gel electrophoresis showing the PCR products of the SCAR marker derived from the species-specific AFLP marker in *T. pagdeni* against genomic DNA of *T. pagdeni* (Lanes 1-18, A and 1-6, B), *T. laeviceps* (Lanes 7B and 1-3, C), *T. spp* (Lanes 4-5, C), *T. thoracica* (lanes 18B and 6C) and *T. fuscobalteata* (Lanes 7-20, C), *T. melanoleuca* (lane 8B), *T. melina* (lane 9B), *T. minor* (lane 10B), *Lisotrigona furva* Engel (lane 11B), *T. terminata* (lane 12B), *T. itama* (lane 13B), *T. canifrons* (lane 14B), *T. collina* (lane 15B), *T. apicalis* (lane 16B), *T. fimbriata* (lane 17B). Lane M and N are a 100 bp ladder and the negative control (without DNA template), respectively.

Table 2.2 Sample sizes of stingless bees used in the species-specific test of the CUTPTP primers

Species	Subgenus	positive result	% of investigated individuals
T. pagdeni	*Tetragonula*	129/129	100
T. fuscobalteata	*Tetragonula*	15/15	100
T. laeviceps	*Tetragonula*	1/12	8.3
T. melina	*Tetragonula*	0/1	0
T. minor	*Tetragonula*	0/15	0
T. collina	*Tretragonilla*	11/112	9.8
T. fimbriata	*Homotrigona*	1/3	33.3
T. melanoleuca	*Tetrigona*	0/1	0
T. apicalis	*Tetrigona*	0/9	0
T. canifrons	*Lophotrigona*	0/1	0
T. itama	*Heterotrigona*	0/5	0
T. thoracica	*Geniotrigona*	0/4	0
T. terminata	*Lepidotrigona*	0/9	0
T. doipaensis	*Lepidotrigona*	0/1	0
Lisotrigona furva Engel	-	0/3	0

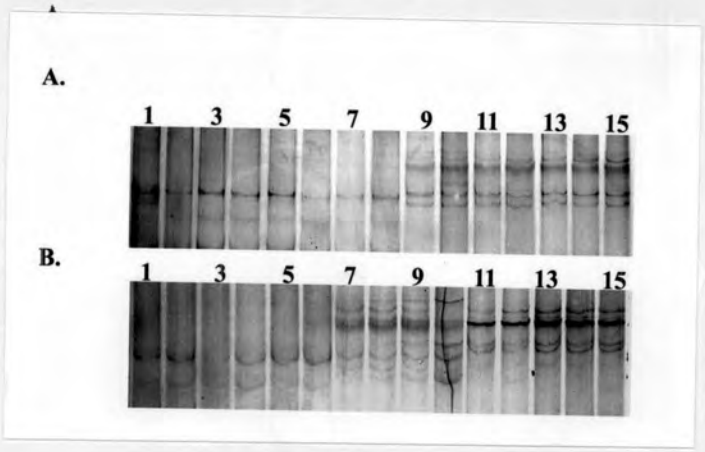

Figure 2.9 SSCP patterns obtained after amplification of *Trigona* DNA with CUTLTP primers (163 bp), *T. pagdeni* (lanes 1–8, A and 1-4, B), *T. fuscobalteata* (lanes 9-15, A and 7-10, B), *T. laeviceps* (lane 5B), *T. fimbriata* (lane 6B) and *T.collina* (lanes 11-15, B).

DISCUSSION

It has been reported by Sakagami (1978) that the Indomalayan stingless bees involved two types, those being easily distinguished from others and those not. The last type included many species of the subgenus *Tetragonula* of the genus *Trigona*. These have been also found in the case of the subgenus *Tetragonula* of the *Trigona* in Thailand. Size variation in *T. laeviceps* was noted earlier. Likewise, size variation in *T. pagdeni* was also recorded in Thailand (Sakagami, 1978). However, our collection data showed many of the species belonging to the taxonomically difficult subgenus *Tetragonula* of the *Trigona* included; *T. pagdeni*, *T. melina*, *T. minor*, *T. laeviceps* and *T. fuscobalteata*.

The remaining *Trigona* species belonging to different subgenus which were easily discriminated from each other by taxonomic identification according to Sakagami and coworkers (1985), were *T. collina* (subgenus *Tretragonilla*), *T. apicalis* (subgenus *Tetrigona*), *T. canifrons* (subgenus *Lophotrigona*), *T. itama* (subgenus *Heterotrigona*), *T. thoracica* (subgenus *Geniotrigona*), *T. fimbriata* (subgenus *Homotrigona*), *T. terminata* (subgenus *Lepidotrigona*), *T. doipaensis* (subgenus *Lepidotrigona*, and *T. melanoleuca* (subgenus *Tetrigona*).

However, the stingless bee species showing the complex morphology can usually be distinguished from each other by using non-morphological data, especially from DNA polymorphism analysis. Fernandes-Salomao and coworkers (2005) examined phylogenetic relationships of eight stingless bee species (*Melipona quadrifasciata anthidioides*, *M. mandacata*, *M. bicolor bicolor*, *M. quinquefasciata*, *M. rufiventis*, *M. scutellaris*, *M. compressipes*, *M. marginata*) using ITS-1 polymorphism. Low sequence divergence between *M. quadrifasciata anthidiodes* and *M. mandacaia* (1.4%) supported that they diverged recently. The phylogenetic relationships derived from sequences of ITS-1 fragments (394-496 bp) were concordant with the taxonomic classification of *Melipona* based on morphological characters.

The stingless bees, *M. quadrifasciata* in Brazil has been divided to 2 subspecies; *M. q. quadrifasciata* which possesses 3-5 continuous yellow stripes and *M. q. anthidiodes*

which possesses 2-5 interrupted stripes on the terca of the 3rd and 6th segments in workers and males. Nevertheless, hybrid individuals exhibiting intermediate stripe patterns were found in some areas. Waldschmidt and coworkers (2000) identified a RAPD fragment (750 bp from OPE07; 5'-AGATGCAGCC-3') that was present in all individuals of *M. q. quadrifasciana* except those from northern Minas Gerais but was absent in *M. q. anthidioides*. This RAPD marker could be used in combination with morphometric data for identification of the hybrid zone of these subspecies. Subsequently, Moretto and Aries (2005) successfully applied PCR-RFLP of mitochondrial DNA genes for differentiation of limited sample sizes of *M. q. quadrifasciata* and *M. q. anthidiodes* (4 colonies from each species). The patterns generated by *Hin*fI- and *Nde*I-digested COI and *Dra*I-digested cytb clearly distinguished these subspecies unambiguously. Notably, mtDNA is haploid and transmitted maternally. Therefore, mtDNA cannot be used to infer the paternal species of hybrid individuals.

Franck and coworkers (2004) studied genetic diversity of the *cabonaria* species group (*Trigona carbonaria*, *T. hockingsi* and *T. davenporti*) from eastern Australia using 13 microsatellite loci. Colonies that displayed as *T. carbonaria* or *T. hockingsi* nest architecture could be unambiguously differentiated at the genetic level but genetically different species (*T. hockingsi* and *T. davenporti*) may have similar nest architectures. The results suggest that nest architectures are efficient but not sufficient to discriminate these species.

In our studies, we applied the AFLP technique that is a popular technique in identify DNA marker in several organisms (Griffiths *et al.*, 2000; Tong *et al.*, 2002). AFLP has been widely used for population genetic studies and identification of molecular markers at different taxonomic levels because the prior knowledge of the sequences of the genome under investigation is not required (Liu and Cordes, 2004). Using 64 primer combinations, a 284 bp fragment generated by *Pst*I$_{+ATC}$/*Mse*I$_{+CAG}$ was solely found in *T. pagdeni*. Nevertheless, AFLP analysis is tedious and time consuming and may cause false positive results when directly applied for authentication of stingless bees species because comigrated AFLP fragments may have different sequences but similar sizes. To

eliminate this possible problem, a candidate *T. pagdeni*-specific AFLP fragment (284 bp) was cloned and sequenced. A pair of sequence-specific primers was designed from this newly unidentified DNA segment (E-value > 10^{-4} for both Blast*N* and Blast*X*, http://ncbi.nlm.nih.gov/blast) and tested across 15 stingless bee species. Of these species in Thailand, *T. fuscobalteata*, *T. laeviceps* and *T. doipaensis*, were not included during screening of AFLP primers.

The expected 163 bp PCR-amplified product was consistently found in *T. pagdeni* (129/129 accounting for 100.0% of investigated individuals; the iridipennis group in subgenus *Tretragonula*) but not in *T. apicalis*, *T. canifrons*, *T. itama*, *T. melina*, *T. minor*, *T. terminata*, *T. doipaensis*, *T. melanoleuca* and *T. thoracica* and the outgroup *Lisotrigona furva* Engel. Nevertheless, cross-species amplification was found in *T. fimbriata* (1/3, 33.3% subgenus *Homotrigona*), *T. collina* (11/112, 9.8%; the atripes group in subgenus *Tretragonilla*), *T. laeviceps* (1/12, 8.3%; the laeviceps group in subgenus *Tretragonula*) and *T. fuscobalteata* (15/15, 100.0%; the iridipennis group in subgenus *Tretragonula*). Therefore, the CUTPTP marker successfully discriminated *T. pagdeni* from 10 stingless bee species and partially differentiated *T. pagdeni* from the other common species *T. collina, T. fimbriata* and *T. laeviceps* but not between *T. fuscobalteata.*

Species-diagnostic markers should be developed from those exhibited low polymorphic (or a fixed pattern) intraspecifically but non-overlapping (or lack of a particular band in the non-target species) interspecifically. SSCP analysis of the CUTPTP marker was then further applied to eliminate false positive problems found in *T. fuscobalteata*, *T. collina*, *T. fimbriata* and *T. laeviceps* and successfully discriminate *T. fuscobalteata* and *T. collina* from other three species. However, differentiation between *T. pagdeni*, *T. collina* and *T. fimbriata* can be unambiguously carried out using their nest architecture and morphology (Sakagami, 1978; Sakagami *et al.*, 1983; Sakagami *et al.*, 1985). Unfortunately, *T. laeviceps* and *T. pagdeni* belong to the same subgenus

Tetragonula of the *Trigona* which show complex and difficult in taxonomic identification.

Differentiation between *T. pagdeni* and *T. laeviceps* samples in this study was later confirmed by Professor Dr. Charles D. Michener (University of Kansas) and deposited in the University of Kansas Snow Entomological Collection. Specimens of *T. laeviceps* had head width of 1.7-1.8 mm, and wing length (measured from apex of alar sclerites to tip of wing) of 3.8-3.9 mm. Specimens of *T. pagdeni* had head width of 1.5-1.6 mm, wing length of 3.1-3.2 mm. The two species showed nearly discrete geographic distributions. *T. laeviceps* was only found at Phrae, Phitsanulok and Pichit provinces in the north population. The two species were sympatric in Phitsanulok province, in nests located near one another. Elsewhere, only *T. pagdeni* was found. *T. laeviceps* and *T. pagdeni* could be discriminated by size and plumose frontal hairs of *T. pagdeni* which disappears in those of *T. laeviceps* (Sakagami, 1978).

In the present study, we demonstrated the successful use of AFLP analysis to generate a candidate species-specific marker of *T. pagdeni*. Polymorphism of the CUTPTP fragment provided reliable molecular markers for discrimination of *T. pagdeni* from *T. fuscobalteata*. Therefore, polymorphism of the CUTPTP marker based on SSCP analysis could be employed to confirm the taxonomic classification of *T. pagdeni* based on morphological characters. It may be useful to differentiate *T. pagdeni* from other *Trigona* species in the iridipennis group which play very similarity in morphology or in the subgenus *Tetragonula* of the *Trigona* which possesses complex in morphology.