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Original article

Genetic diversity and population structure of *Trigona pagdeni* Schwarz in Thailand*

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Abstract – Samples of the stingless bee *Trigona pagdeni* Schwarz were collected from north, northeast, central and peninsular Thailand. Genetic variation and population structure were investigated using a DNA fingerprinting technique, TE-AFLP, and Analysis of Molecular Variance (AMOVA). We found high levels of genetic variation among individuals in all populations, but mean expected heterozygosity was highest in the Northeast. We used AMOVA to calculate ϕ_{PT} (analogous to Wright's ϕ_{ST} for codominant markers) to compare genetic differentiation among populations. This revealed significant genetic differentiation among the four populations (ϕ_{PT} = 0.18, P = 0.01). We also detected differentiation (ϕ_{PT} = 0.13, P = 0.001) between samples collected north and south of the Kra ecotone, a biogeographical zone of transition between seasonal evergreen and mixed moist deciduous forests. However the greatest differentiation was detected between samples from the northeast and the other locations combined (ϕ_{PT} = 0.21, P = 0.001). This method can be applied to the study of population structure in *T. pagdeni* and other stingless bees, and may provide a useful tool for management and conservation of this species.

Meliponini / *Trigona* / DNA fingerprinting / AFLP / geographic variation

1. INTRODUCTION

Stingless bees of the genus *Trigona* Jure 1807 (Apidae, Apinae, Meliponini) occur in tropical and subtropical regions worldwide, where they are important pollinators (Michener, 1974, 1990, 2000; Sakagami, 1982). Thirty two species of *Trigona* are known from Thailand (Schwarz, 1939; Sakagami et al., 1985; Michener and Boongird, 2004; Klakasikorn et al., 2005). *Trigona pagdeni* Schwarz is a common stingless bee known from Thailand, peninsular Malaysia and the Indochina region (Sakagami, 1978; Sakagami and Khoo, 1987). Our goal is to survey genetic diversity and population structure of *T. pagdeni* in Thailand.

Earlier studies of *Apis cerana* in Thailand using mitochondrial DNA (Smith and Hagen, 1996, 1999; Sihanuntavong et al., 1999; Warrit et al., 2006) and microsatellites (Sittipraneed et al., 2001) showed genetic differentiation among geographic regions within Thailand,

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particularly between populations north and south of 11°N in the Isthmus of Kra (Warrit et al., 2006). This region, known as the Kra ecotone, corresponds to the transition between seasonal evergreen or seasonal rainforest and mixed moist deciduous or monsoon forest (Hughes et al., 2003), and between the Indochinese and Sundaic biotas (Woodruff, 2003). Shifts from one species or subspecies to another north and south of this ecotone have been documented in many animal taxa as well (e.g., birds). However no such information exists for the numerous stingless bee species of Thailand; therefore the goal of this paper is to investigate the geographic pattern of genetic diversity in *T. pagdeni*, and to determine if similar biogeographic patterns are shown by both *A. cerana* and stingless bees. This information will help to establish the generality of the Kra ecotone as a biogeographic boundary between northern and southern populations of bees in the Thai-Malay peninsula, and will also be fundamental for establishing rational management of resources for conservation of this native species.

We chose a DNA fingerprinting technique, Three-Enzyme Amplified Fragment Length Polymorphisms (TE-AFLP: van der Wurff et al., 2000) to survey genetic diversity because it reveals intraspecific variation across the genome, requires no prior knowledge of genome sequences, and is relatively rapid and inexpensive.

2. MATERIALS AND METHODS

2.1. Sampling

Adult worker stingless bees were collected from 117 nests of *T. pagdeni* at the localities shown in Figure 1 and Table I. Specimens were placed in 95% ethanol and kept at 4 °C until required. Taxonomic identification was carried out according to Sakagami (1978) and Sakagami et al. (1985, 1990), and later confirmed by Dr. C.D. Michener (University of Kansas). Voucher specimens of *T. pagdeni* from some of the colonies used in this study are deposited in the University of Kansas Snow Entomological Collection.

2.2. DNA extraction

Total DNA was extracted from one entire bee per nest using standard proteinase K-SDS digestion of tissue followed by phenol-chloroform extraction and ethanol precipitation (Smith and Hagen, 1996). DNA concentration was estimated by comparison with a known quantity of digested lambda DNA on a mini-gel (Sambrook et al., 1989).

2.3. TE-AFLP Fingerprinting

Approximately 30 ng of each DNA extract was digested with the 6-base restriction enzymes *Xba*I and *Bam*HI and the 4-base enzyme *Rsa*I in a 20 μL reaction. The resulting fragments were ligated to linkers (described in van der Wurff et al., 2000) having sticky ends complementary to the *Xba*I and *Bam*HI sticky ends of the DNA fragments. Next, 0.5 μL of the digestion-ligation mix was used as template in a 12.75 μL PCR reaction containing 2.5 pM each of unlabeled *Xba*I-primer and ^{32}P-labelled *Bam*HI primer, 1X PCR buffer (10 mM Tris-HCl, pH 7.6, 50 mM KCl, 0.1% Triton X-100 and 1.5 mM $MgCl_2$), 50 μM of each dNTP (Promega), and 0.6 U *Taq* DNA polymerase (Promega). The *Xba*I and *Bam*HI primer sequences (van der Wurff et al., 2000) are identical to one strand of the *Xba*I and *Bam*HI linkers, respectively, with arbitrary base extensions. We used a labeled *Bam*HI primer with the arbitrary extension "C", and paired it with two different *Xba*I primers: *Xba*I-CC and *Xba*I-AC.

The polymerase chain reaction was performed on a thermal cycler (TC-412; TECHNE) using 2.5 min denaturation at 95 °C followed by: 10 cycles of 30 s denaturation at 95 °C, 30 s annealing at 70 °C, 60 s elongation at 72 °C, and 40 cycles of 30 s denaturation at 95 °C, 30 s annealing at 60 °C and 60 s elongation at 72 °C, with a final 20 min at 72 °C (van der Wurff et al., 2000). For analysis, the PCR product was mixed with 3 μL loading dye (formamide containing 5 mg/mL blue dextran and 2.4 μL of sterile double-distilled water) and denatured for 5 min at 95 °C. Three μL was loaded on a 8% denaturing polyacrylamide gel with 0.6x TBE electrophoresis buffer and electrophoresed for 5–6 h at 1000 V. The dried gel was used to expose Kodak X-O1000 mat film.

2.4. Scoring TE-AFLP variation

Bands generated by each primer combination were scored by eye. Presence of a TE-AFLP band

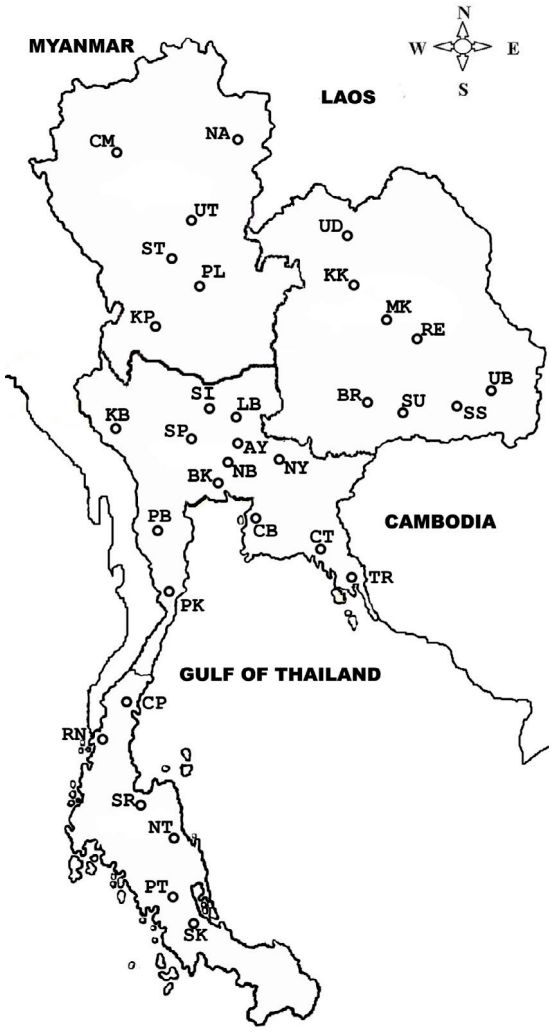

Figure 1. Collection sites of *Trigona pagdeni* Schwarz in Thailand. See Table I for locality names and sample sizes.

was scored as 1, absence as 0. Thus each bee was characterized by a multi-locus phenotype.

2.5. Data analysis

Individuals were grouped into four populations based on collection site: North, Northeast, Central and Peninsula (Fig. 1, Tab. I). Genetic diversity, genetic distance among populations, and population structure statistics were calculated using Genetic Analysis in Excel (GenAlEx6; Peakall and Smouse, 2006).

Genetic diversity was estimated as the proportion of polymorphic loci (P), and expected heterozygosity (H_e). A band was considered polymorphic if our samples showed any variation for presence or absence, though "singletons" (bands present or absent in only a single individual) and other rare variants have little effect on assessment of population structure. Estimation of H_e followed the method of Lynch and Milligan (1994), which considers each band position as a different locus with

Table I. Collection sites, population, map abbreviation (Fig. 1) and sample sizes of *Trigona pagdeni* used in this study.

Localities	Population	Map Abbreviations	Sample size
Nan	North	NA	2
Uttaradit	North	UT	1
Kamphaeng Phet	North	KP	1
Phitsanulok	North	PL	2
Sukhothai	North	ST	5
Chiang Mai	North	CM	1
Udon Thani	Northeast	UD	1
Khon Kaen	Northeast	KK	3
Ubon Rachathani	Northeast	UB	3
Sisaket	Northeast	SS	1
Roi Et	Northeast	RE	6
Surin	Northeast	SU	4
Buriram	Northeast	BR	2
Maha Sarakham	Northeast	MK	3
Nonthaburi	Central	NB	1
Lopburi	Central	LB	2
Suphanburi	Central	SP	1
Sing Buri	Central	SI	2
Nakhon Nayok	Central	NY	1
Bangkok	Central	BK	1
Ayutthaya	Central	AY	3
Phetchaburi	Central	PB	1
Kanchanaburi	Central	KB	10
Prachuap Khiri Khan	Central	PK	15
Trat	Central	TR	2
Chanthaburi	Central	CT	3
Chonburi	Central	CB	2
Chumphon	Peninsular	CP	17
Ranong	Peninsular	RN	1
Nakhon Si Thammarat	Peninsular	NT	1
Surat Thani	Peninsular	SR	6
Phattalung	Peninsular	PT	4
Songkhla	Peninsular	SK	9

two alleles, band amplified (dominant) and band not amplified (recessive); absence of a band indicates a recessive homozygote. At each locus, the frequency of the recessive allele (q) is estimated from the frequency of putative recessive homozygotes (q^2), and the frequency of the dominant allele is estimated as: $p = 1 - q$. Expected heterozygosity at each locus = $h = 1 - \Sigma\, x_i^2$, where x_i is the frequency of the *i*th allele, or in this case, $1 - (p^2+q^2)$. Expected heterozygosity averaged over all loci was calculated as:

$$H_e = 1 - 1/m \sum_{y=1}^{m} \Sigma x_i^2 \qquad (1)$$

where *y* represents loci or bands 1 through *m*.

Analysis of Molecular Variance (AMOVA; Excoffier et al., 1992) implemented in GenAlEx (Peakall and Smouse, 2006) was used to assess the patterns of observed genetic variation. AMOVA can be used with binary or dominant markers such as TE-AFLPs to calculate ϕ statistics, which are analogous to Wright's F-statistics for co-dominant data (Wright, 1951, 1965). ϕ_{PT}, which can take values from 0–1, measures the similarity of pairs of individuals drawn at random from the same population, relative to pairs of individuals drawn from the entire collection, and is analogous to Wright's F_{ST}.

In order to search the data set for geographic patterns, we examined (1) differentiation among the four geographic populations and (2)

differentiation between Peninsula samples and those from north of the Isthmus of Kra. Because mean expected heterozygosity and pairwise genetic distances among the four populations indicated the Northeast population differed from the others, we also examined (3) differentiation between Northeast samples and the others. In each case, significance testing was performed by comparing observed statistics to the distributions generated by 999 random permutations of the data into arbitrary populations of the same size as the actual geographic populations.

3. RESULTS

3.1. Phenotypic diversity

The presence or absence of 51 TE-AFLP bands was scored for each individual. Eight bands (16%) were fixed (present in all 117 *T. pagdeni*). The remaining 43 bands, each of which was present in at least one individual *and* absent in at least one individual, were considered polymorphic. Thirty five of these bands (69%) had inferred allele frequencies in the range of 5% to 95%. The mean expected heterozygosity, H_e, was 0.195 (st. error 0.022). We observed 87 unique banding patterns or phenotypes for the primer pair *Bam*HI-C and *Xba*I-CC, and 77 for the primer pair *Bam*HI-C and *Xba*I-AC (data not shown). Sample banding patterns are shown in Figure 2.

3.2. Population structure

Table II compares genetic diversity in the four populations of *T. pagdeni*. Within populations, 14–19 bands were fixed. Two bands were found only in the Northeast population; no other population showed "private" bands (data not shown). The Northeast population shows the highest percentage of polymorphic bands and highest mean expected heterozygosity.

Pairwise genetic distances among populations are shown in Table III; the highest values are those comparing the Northeast population to others. AMOVA results are shown in Tables IV and V. Differentiation was observed among all four populations ($\phi_{PT} = 0.18$, $P = 0.01$), though 82% of observed variation occurred among individuals within populations. If bees collected north of the Kra ecotone are combined into a single population, small but significant differentiation between bees north and south of the Kra ecotone can be detected ($\phi_{PT} = 0.13$, $P = 0.001$). However, the greatest differentiation was observed in the contrast of bees from the Northeast with the other combined samples ($\phi_{PT} = 0.21$, $P = 0.001$).

4. DISCUSSION

DNA fingerprinting (TE-AFLPs) revealed substantial genetic variation among *T. pagdeni* collected throughout Thailand. Analysis of population structure showed differentiation among the Peninsula, Central, North and Northeast population, and between *T. pagdeni* north and south of the Kra ecotone. However, the greatest differentiation was between *T. pagdeni* from the Northeast and the other samples ($\phi_{PT} = 0.21$, $P = 0.001$, Tab. V); we also found higher pairwise genetic distances between the Northeast population and each of the other three populations, than between other pairs of populations (Tab. III). The samples from the Northeast region also show a higher level of polymorphism and expected heterozygosity compared to other samples (Tab. II).

This pattern seems to be different from that found for *Apis cerana*, the bee species which has been most thoroughly investigated in Thailand. DNA sequence data (e.g., Warrit et al. 2006; Smith and Hagen, 1996) indicate that the Kra ecotone marks a major shift in mitochondrial lineages of *A. cerana*. Microsatellite data (Sittipraneed et al., 2001) also showed differences between populations of *A. cerana* north and south of this ecotone, though no sharp boundaries between northern and southern lineages were observed. The most direct comparison of *T. pagdeni* and *A. cerana* would use the same data set for both species, but published data do not yet exist for either TE-AFLPs for Thai *A. cerana*, or mitochondrial sequences for *T. pagdeni*. Furthermore, *A. cerana* from the Northeastern region have not been well-sampled yet, so we cannot say whether they

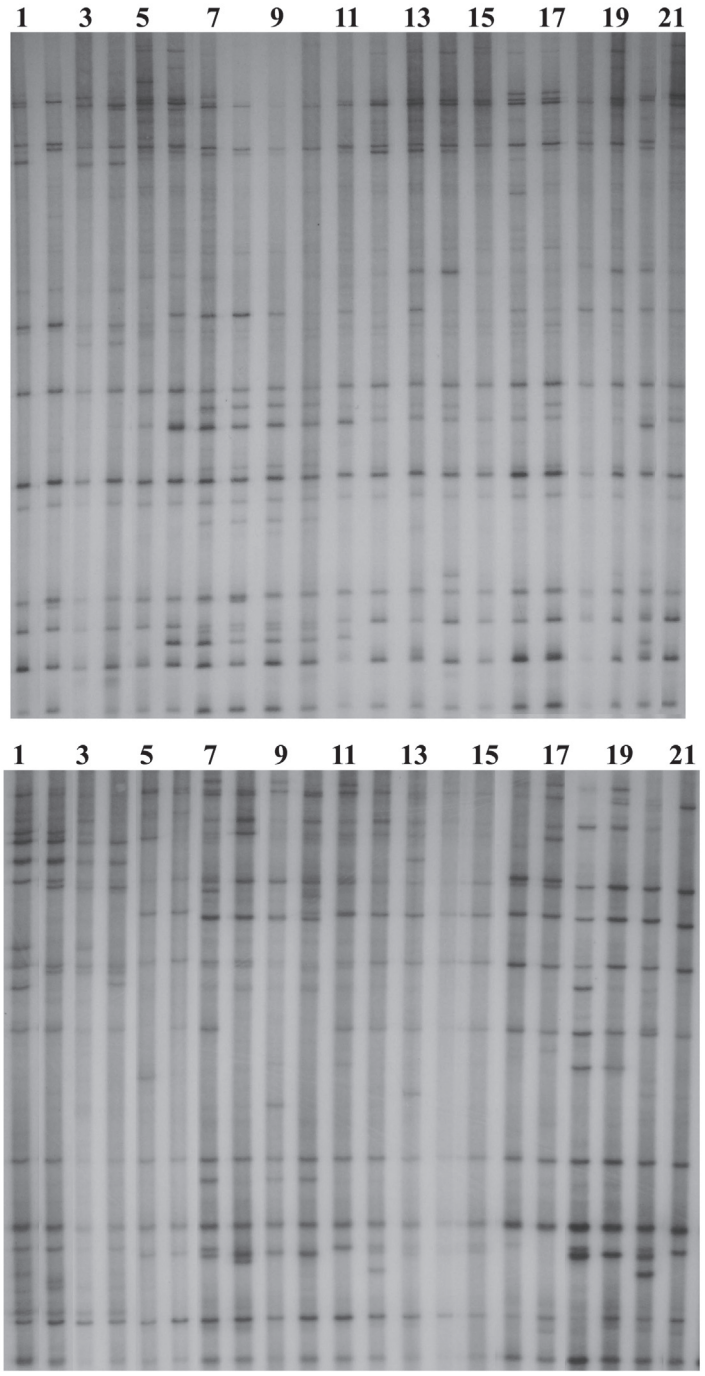

Figure 2. Autoradiograph of part of a TE-AFLP pattern obtained after amplification of *Trigona* DNA with primers (Top) *Bam*HI-C and *Xba*I-CC and (Bottom) *Bam*HI-C and *Xba*I-AC. Lanes 1–4 are a related species, *T. laeviceps*, lanes 5–21 are *T. pagdeni*; (lane 5–6, North population; lanes 7–11, Northeast population; lanes 12–17, Central population; and lanes 18–21, Peninsular population).

Table II. Comparison of 51 TE-AFLP bands generated by primer pairs *Bam*HI-C and *Xba*I-CC, and *Bam*HI-C and *Xba*I-AC in four populations of *Trigona pagdeni* in Thailand. Fixed bands were present in every individual sampled, polymorphic bands include any band that is present in at least one individual *and* absent in at least one individual. Allele frequencies are inferred from the frequency of individuals in which a band is absent (considered recessive homozygotes) assuming alleles are in Hardy-Weinberg equilibrium (Lynch and Milligan, 1994).

	Locality (N bees)			
Bands:	NORTH (12)	CENTRAL (44)	NORTHEAST (23)	PENINSULA (38)
Number scored	41	45	48	43
Number fixed	19	19	14	17
Number absent	10	6	3	8
# polymorphic	22	26	34	26
% polymorphic	54% of 41 (43% of 51)	58% of 45 (51% of 51)	71% of 48 (67% of 51)	60% of 43 (51% of 51)
Loci:				
# loci with inferred allele frequencies 5–95%	16	23	28	19
% with inferred allele frequencies 5–95%	39% of 41 (31% of 51)	51% of 45 45% of 51	58% of 48 55% of 51	44% of 43 37% of 51
mean *He* (std. error)	0.130 (0.026)	0.137 (0.025)	0.207 (0.207)	0.139 (0.026)

Table III. Pair-wise genetic distances among populations of *Trigona pagdeni* in Thailand, as estimated in GenAlEx6 (Peakall and Smouse, 2006).

	North	Northeast	Central	Peninsula
North (N = 12)	*			
Northeast (N = 23)	0.061	*		
Central (N = 44)	0.018	0.057	*	
Peninsula (N = 38)	0.029	0.079	0.033	*

are conspicuously diverse or strongly differentiated from other Thai *A. cerana* populations. However, it is known that *A. cerana* from neighboring Cambodia and southern Vietnam have more diverse mitochondrial haplotypes than populations found in North, Central and Peninsular Thailand (Smith et al., 2005).

In this study we showed that the TE-AFLP procedure can be used to investigate genetic diversity and population structure of *Trigona* bees. This may provide useful information for management and conservation of this important pollinator of tropical plants, whose

Table IV. Analysis of Molecular Variance (AMOVA) of TE-AFLP banding patterns generated by two primer pairs in 117 *Trigona pagdeni* from 4 population groupings (North, Northeast, Central and Peninsula). df = degrees of freedom, SS = sums of squares, MS = mean squares, Est. Var. = estimated variance within and among populations, % = percentage of observed variance within or among populations, ϕ_{PT} = correlation of individuals within populations relative to the collection as a whole. Significance testing was carried out by comparison of observed values to those generated by 999 permutations in which individuals were randomly assigned to four populations of the same size as the four study populations. Prob. = the probability of obtaining a value of ϕ_{PT} as large or larger when individuals are randomly assigned to groups.

	df	SS	MS	Est. Var.	%	ϕ_{PT} (P)
Among Populations	3	80.47	6.82	0.84	18%	
Within Populations	113	428.30	3.79	3.79	82%	0.18 (P = 0.01)

Table V. AMOVA of TE-AFLP banding patterns generated by two primer pairs in 117 *Trigona pagdeni*. Upper chart (A): samples grouped in two populations: Northeast and Others; Lower chart (B) samples grouped into two populations: South (same as Peninsula population) and North of the Kra ecotone. Significance testing by comparison of observed values to those generated by 999 random permutations of individuals into populations of the same size. Prob. = the probability of obtaining a value of ϕ_{PT} as large or larger when individuals are randomly assigned to groups. Other abbreviations are as in Table IV.

		df	SS	MS	Est. Var.	%	ϕ_{PT} (P)
A. Northeast vs. others	Among Populations	1	44.11	44.11	1.08	21%	
	Within Populations	115	464.66	4.04	4.04	79%	0.21 (P = 0.001)
B. North vs. South of Kra Ecotone	Among Populations	1	35.84	35.84	0.62	13%	
	Within Populations	115	472.93	4.11	4.11	87%	0.13 (P = 0.001)

original habitat is decreasing because of human development (see, for example, Araujo et al., 2004). For example, it has recently become popular to cultivate *T. pagdeni* in boxes for plant pollination, and human transportation of such hives could lead to mixing of genetically differentiated populations.

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Diversité génétique et structure de la population de *Trigona pagdeni* Schwarz en Thaïlande.

Meliponini / *Trigona* / abeille sans aiguillon / variation géographique / empreinte génétique ADN / AFLP / Thaïlande

Zusammenfassung – Genetische Diversität und Populationsstruktur von *Trigona pagdeni* Schwarz in Thailand. Die Stachellose Biene *Trigona pagdeni* Schwarz (Apidae, Apinae, Meliponini) ist eine der häufigsten Stachellosen Bienen in Thailand. Wir untersuchten die genetische Diversität und Populationsstruktur von *T. pagdeni* in Thailand mittels einer DNA Fingerprinting Metodologie, der Drei-Enzym-Amplifikationsfragment-Längenpolymorphismus Methode (TE-AFLP, van der Wurff et al., 2000), da sie intraspezifische Genomvariabilität aufdeckt, ohne dass Vorkenntisse zur Genomstruktur erforderlich sind, und da sie ausserdem relativ schnell und kostengünstig zu Ergebnissen führt. Wir zeigen, dass die TE-AFLP-Methode für Untersuchungen zur genetischen Diversität und Populationsstruktur von *Trigona*-Bienen geeignet ist.

Wir sammelten Arbeiterinnen von 117 *T. pagdeni* Nestern an den in Abbildung 1 und Tabelle I. aufgelisteten Standorten und bewahrten diese in 95 % Ethanol bei 4 °C auf. Die DNA-Extraktion erfolgte mittels Standardmethoden (Smith and Hagen, 1996). Die TE-AFLP Methode folgte dem Protokoll von van der Wurff et al. (2000). Wir benutzten zwei Primerpaare: einen ^{32}P-markierten *Bam*HI Primer mit einer "CNukleotidzufallsverlängerung, gepaart mit einem *Xba*I Primer mit -CC or -AC Nukleotidzufallsverlängerungen. Die amplifizierten Fragmente wurden in 8 % denaturierenden Polyacrylamidgelen elektrophoretisch bei 500 V in 5–6 h aufgetrennt. Die getrockneten Gele wurden auf Röntgenfilmen exponiert und entwickelt. Banden wurden als anwesend (1) oder abwesend (0) gewertet, so dass jede Biene mittels eines Multilocus-Phänotyps charakterisierbar war. Eine Bande wurde als polymorph bewertet, wenn sie in unseren Proben eine Variation in punkto anwesend/abwesend zeigte.

Die Individuen waren in vier Gruppierungen entsprechend der Sammelorte Norden, Nordosten, Zentrum und Halbinsel auftrennbar (Abb. 1, Tab. I). Der Prozentsatz polymorpher Loci,

erwarteter Heterozygotiegrad, genetische Distanz zwischen Populationen und Statistiken zur Populationsstruktur wurden mittels des Programms Genetic Analysis in Excel (GenAlExV6.1; Peakall and Smouse, 2006) berechnet. Wir benutzten AMOVA (Analysis of Molecular Variance; Excoffier et al., 1992), um den Differenzierungsgrad zwischen Populationen zu bewerten. Da *Apis cerana* und auch andere Taxa eine genetische Differenzierung zwischen Populationen nördlich und südlich des 11. Breitengrads N auf dem Isthmus von Kra aufweisen (Hughes et al., 2003; Woodruff, 2003; Warrit et al., 2006), untersuchten wir Anhaltspunkte dafür auch in unseren *T. pagdeni* Proben. Basierend auf den genetischen Distanzen zwischen den Populationen testen wir auch, ob sich die Proben aus dem Nordosten von allen anderen unterscheiden.

Tabelle II vergleicht die genetische Diversität für die vier Populationen von *T. pagdeni*. Der Prozentsatz polymorpher Loci und der erwartete Heterozygotiegrad war am höchsten in Proben aus dem Nordosten. Tabelle III zeigt die paarweisen genetischen Distanzen zwischen den vier *T. pagdeni* Populationen. Die paarweise genetische Distanz zur Population aus dem Nordosten war grösser als die zwischen allen anderen Populationen untereinander. Die AMOVA Ergebnisse sind in den Tabellen IV und V zusammengestellt. Diese zeigen eine Differenzierung zwischen allen vier Populationen (ϕ_{PT} = 0,18, P = 0,01), und eine Differenzierung für Bienen nördlich und südlich der Kra Ecotone (ϕ_{PT} = 0,13, P = 0,001). Der deutlichste Kontrast war allerdings für Bienen aus dem Nordosten im Vergleich zu allen anderen Populationen zu sehen, die damit für einen Grossteil der beobachteten genetischen Variabilität verantwortlich sind (ϕ_{PT} = 0,21, P = 0,001). In diesem Punkt unterscheiden sich unsere Ergebnisse von den Mustern, die für mitochondriale Sequenzen von *A. cerana* vorliegen.

Meliponini / *Trigona* / DNA Fingerprinting / AFLP / geographische Variation

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