MtDNA variation among subspecies of *Apis cerana* using restriction fragment length polymorphism

S Deowanish 1, J Nakamura 1, M Matsuka 1*, K Kimura 2

1 Honeybee Science Research Center, Tamagawa University, Machida, Tokyo, 194;
2 National Institute of Animal Industry, Tsukuba Norindanchi PO Box 5, Ibaraki, 305 Japan

(Received 11 September 1996; accepted 8 November 1996)

Summary — Mitochondrial DNA variation of *Apis cerana* from Japan, Korea, Taiwan, Vietnam, Thailand, Nepal and the Philippines was examined by RFLP analysis. Using ten restriction enzymes, we could discriminate among different localities including groups from: (1) Japan; (2) Nepal, Vietnam and north-to-central Thailand; (3) Korea-Tsushima; (4) Taiwan; (5) south Thailand; and (6) Philippines.

*Apis cerana* / mtDNA / RFLP / variability

INTRODUCTION

*Apis cerana* Fabr, a honeybee native to Asia, was classified into four subspecies by Ruttner (1988) on the basis of morphometric and geographic distribution; *Apis cerana himalaya* ranges the south-east Asian mountains through Nepal to Thailand and probably south-west China. *Apis cerana* ranges through Afghanistan, Pakistan, north India, northern and eastern China and north Vietnam. *Apis cerana indica* occurs from south India, Sri Lanka, Bangladesh, Myanmar, Malaysia, southern Thailand, Indonesia and the Philippines, while *Apis cerana japonica* is restricted to Japan.

MtDNA analysis has proved to be a powerful tool for identifying population diversity among subspecies of the western honeybee (*Apis mellifera* L). Restriction fragment length polymorphisms (RFLPs) in mtDNA have been used to distinguish between African and European races (Moritz et al, 1986; Smith, 1988; Smith and Brown, 1990; Sheppard et al, 1991a,b). MtDNA restriction site maps have been constructed for many subspecies (Smith, 1988; Smith and Brown, 1990; Garnery et al, 1991, 1992). Length polymorphism in the region between cytochrome oxidase (CO I and CO II) genes in mtDNA in *A mellifera* was reported by Cornuet et al (1991). The complete sequence of mtDNA of *A mellifera* was reported by Crozier and Crozier (1993). Previous molecular data on the evolutionary history of honeybees inferred from mtDNA analysis indicates that *A cerana* is the clos-
est relative to *A mellifera* (Garnery et al., 1991, 1992).

Variation in mtDNA of *A cerana* was first studied by Smith (1991, 1993) who proposed three groups: a mainland Asian group including Japan, Thailand, Malaysia, Borneo and south India; a group from Luzon; and a group from the Andaman Islands. This result differed from the morphological identification (Ruttner, 1988) which separates *A c japonica* from the other groups. Since the mtDNA study examined few samples from some locations and no samples from the vast region of China and the Himalayas, it cannot be expected to fully explain phylogenetic relationship of *A cerana* populations.

We investigated mtDNA variation among *A cerana* from 22 different locations using RFLPs, by digesting with ten restriction enzymes followed by hybridization with a probe from PCR-amplified product between tRNA^Leu^ and CO II of mtDNA of *A c japonica*.

**MATERIALS AND METHODS**

**Sample collection**

Adult honeybees (20–30) were collected from 27 colonies of *A cerana* at 22 locations (fig 1), immersed in absolute ethanol or immediately frozen with dry ice or liquid nitrogen and stored at -70 °C until DNA extraction.

**DNA extraction, digestion and Southern-blot hybridization**

The total DNA from each sample was extracted from the thoraxes of the honeybees, generally following the procedure of Lee (1993), and digested using ten restriction enzymes (Hae III, Hinf I (four-bases enzymes), Bcl I, Bgl II, EcoRI, EcoRV, Hinc II, Hind III, Nde I and Spe I (six-bases enzymes)) according to the manufacturer's instruction (Takara). Restriction fragments were separated on 1% agarose gels. After staining with ethidium bromide and detection under UV light, DNA fragments on the gel were Southern-blot-transferred to nylon membrane (Nytran, Schleicher and Schuell) (Maniatis et al, 1982) and hybridized with a labeled probe. The probe consisted of PCR-amplified product obtained from the use of two primers, E2 and H2 as described by Garnery et al (1991) (30 temperature cycles of 1 min at 95 °C, 1 min at 55 °C and 1 min at 72 °C). In A mellifera this amplification product includes the mtDNA region between tRNALeu and the CO II region. The probe was labeled using a random primed labeling Kit (Boehringer, Mannheim). The hybridized fragments were detected using a DIG chemiluminescent detection system (Boehringer, Mannheim) and the results were visualized on X-ray film.

**Data analysis**

The data for hybridized fragments were organized into 1-0 matrix and analyzed using Statistica (StatSoft, Inc), Euclidean distance was calculated to provide an estimate of genetic distance and the phenogram was constructed by the unweighted pair group method with arithmetic mean (UPGMEA).

**RESULTS AND DISCUSSION**

Several RFLP patterns were obtained using the ten restriction enzymes (table I). Examples are shown in figure 2. Hae III showed ten patterns from all samples. With this enzyme, Japanese honeybees from Honshu had a different pattern from the other locations. Two Hinc II patterns were detected, and Philippines bees could be separated from the others by this enzymes. Hind III showed three patterns and bees from south Thailand could be separated from the others. Three EcoRV patterns were detected and honeybees from Honshu and south Thailand could be separated.

No difference was found between patterns for Japanese honeybee samples from Honshu. However, two samples from Tsushima showed different patterns from the Honshu samples and were similar to the Korean samples when digested with EcoRI, Hae III, Nde I and Spe I.

Although no variation in mtDNA patterns was found among Nepalese bees, their pattern was similar to three Vietnamese sam-

![Fig 2. Fragments of mtDNA of A cerana after digestion with EcoRV and hybridized with labeled probe from tRNALeu to CO II region.](image)
Within Vietnamese bees, one colony from Hanoi differed from the others when digested with EcoR I and Hae III, and two colonies from Hanoi differed from the others when digested with Hinf I.

Variation in mtDNA of *A. cerana* from Taiwan was found when digested with Bgl II and Hae III, but there was no variation with other enzymes. Taiwanese bees as a whole were separated from the other locations when digested with Bcl I, EcoR I and Hinf I.

Variation in mtDNA among Thai samples was found with Bgl II, EcoR V, Hae III, Hinf I and Nde I; samples from the south (Hatyai and Samui) were separated from the others when digested with EcoR V and Hind III.

The clustering analysis (fig 3) produced by this study divided *A. cerana* from the different locations into six groups: (1) a Japanese group; (2) a Nepal, Vietnam and north-to-central Thailand group; (3) a Korea-Tushima group; (4) a Taiwan group; (5) a south Thailand group; and (6) a Philippines group.

Previous studies using five restriction enzymes (Bcl I, Bgl II, EcoR I, Hind III and Spe I) by Smith (1991, 1993) indicated that *A. c japonica* was included in the mainland Asia group. When we investigated with the same enzymes, *A. c japonica* from Honshu was still included in the Nepal, Vietnam and Thailand groups, while *A. cerana* from Tsushima (located between Korea and Japan) showed different patterns from Honshu, and Thai bees showed differences within Thailand. However, additional restriction enzymes (Hae III, Hinf I, EcoR V, Hinc II and Nde I), revealed differences between *A. c japonica* from Honshu and *A. cerana* from the other locations. Similarly, samples from south Thailand could be separated from those of northern Thailand. These results are in accordance with the morpho-

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**Table I. Numbers of distinct bands and numbers of patterns recognized.**

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<tr>
<th>Enzymes</th>
<th>No of bands</th>
<th>No of patterns</th>
<th>Notes</th>
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<tr>
<td>Hae III</td>
<td>10</td>
<td>10</td>
<td>Honshu group could be separated, high level of variation in Taiwan and Thailand</td>
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<tr>
<td>Hinf I</td>
<td>10</td>
<td>10</td>
<td>Honshu, Nepal, Thailand and Vietnam bees in group while others could be separated</td>
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<tr>
<td>Bcl I</td>
<td>6</td>
<td>4</td>
<td>Variation within Taiwanese bees found</td>
</tr>
<tr>
<td>Bgl II</td>
<td>6</td>
<td>5</td>
<td>Korea-Tushima group, Taiwan group, and Philippines distinguished from each other</td>
</tr>
<tr>
<td>EcoR I</td>
<td>6</td>
<td>5</td>
<td>Honshu group and south Thailand group could be separated</td>
</tr>
<tr>
<td>EcoR V</td>
<td>5</td>
<td>3</td>
<td>Philippines could be separated</td>
</tr>
<tr>
<td>Hind III</td>
<td>2</td>
<td>2</td>
<td>South Thailand group could be separated</td>
</tr>
<tr>
<td>Nde I</td>
<td>3</td>
<td>3</td>
<td>Samples from Philippines, Korea-Tushima showed same patterns but different from others groups</td>
</tr>
<tr>
<td>Spe I</td>
<td>2</td>
<td>2</td>
<td>Samples from Philippines, Korea-Tushima showed same patterns but different from others groups</td>
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These data indicate that using a probe from the intergenic region of *A. mellifera* mtDNA from tRNA<sub>Leu</sub> to CO II can distinguish genetic variation among *A. cerana*. As expected, Hae III and Hinf I (four-base restriction enzymes) showed more variation than six-base restriction enzymes. However, the six-base enzymes EcoRV and Hind III were useful for separating Japanese honeybees and bees from south Thailand. The similarity of *A. cerana* from Tsushima to Korean rather than Japanese honeybees is of interest in the spread of *A. cerana* in these areas. Perhaps *A. cerana* was introduced from Korea to Tsushima by beekeepers.

Although the sample treatment was limited, these results indicate that the method we used in this study is useful and show additional variation for analysis of population structure and genetic relationships within *A. cerana*. However, further studies with finer scale and larger samples are needed before conclusions about the phylogeographic distribution can be drawn.

ACKNOWLEDGMENTS

We wish to thank to Y Obara and T Satoh, Tokyo University of Agriculture and Technology, for their advice, facilities and materials in the initial stage of this research. We wish to thank K Woo, Seoul National University and CR Cervancia, University of Philippines for providing honeybee samples from Korea and the Philippines, respectively. We also thank T Yoshida, M Sasaki and K Takeuchi, Tamagawa University, for their help in collecting samples.

_Apis cerana_ den _Apis cerana_ Gruppen des südostasiatischen Festlande sehr ähnlich
ist. Sie schlug deshalb 3 Unterarten vor. Von
27 _Apis cerana_ Völkern wurden jeweils 20-30
adulte Honigbienen an 22 verschiedenen
Orten gesammelt (Abb 1). Die Proben wur-
den in absolutem Alkohol konserviert oder
sofort mit Trockeneis oder flüssigem Stick-
stoff eingefroren und bei −70 °C für die spä-
tere Analyse aufbewahrt. DNA von jeder
Probe wurde mit 10 Restriktionsenzymen
verdaut (_Hae_ III, _Hinf_ I (ein vier – Basen –
Enzym), _Boll, Bgl_, _EcoRI, EcoRV, Hinc II,
_Hind_ III, _Nde_ I and _Spe_ I (ein sechs – Basen –
Enzym). Die Restriktionsfragmente wur-
den in einem 1% Agarose Gel aufgetrennt
und durch Southern-Blotting auf eine Nylon-
membran übertragen. Das durch PCR ver-
vießfältigte Produkt der mtDNA von _A c japonica_ (zwischen der tRNA Leu und der CO II
Region) wurde markiert und als Sonde für
die Hybridisierung benutzt. Hybridisierte Ele-
mente wurden sichtbar gemacht und die
Unterschiede im Bandenmuster analysiert.
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Die größere Ähnlichkeit von _A cerana_ abdeckt, benötigt,
bevor man über die Verwandtschaft dieser
Population Aussagen machen kann.

_Resumen — Étude de la variation de
l'ADNmt chez les sous-espèces d'Apis
cerana à l'aide du polymorphisme de lon-
gueur des fragments de restriction
(RFLP)._ 

_Apis cerana_ a été séparé par Ruttner
(1988) en quatre sous-espèces en fonction
de la morphométrie et de la répartition géo-
de la variation de l'ADNmt a montré que les
génomes mitochondriaux d _A cerana_ japo-
nica sont apparentés à ceux d _A cerana_ du sud-est asiatique, ce qui suggère l'existence
de trois groupes. Des abeilles adultes
(20-30) ont été prélevées dans 27 ruches réparties dans
22 localités différentes (fig 1). Les échantillons ont été immergés dans de
l'éthanol absolu ou immédiatement conge-
lés dans de la carboglace ou de l'azote
liquide et stockés à −70 °C pour les ana-
lyses ultérieures d'ADNmt. L'ADN de
chaque échantillon a été digéré par dix
enzymes de restriction : _Hae_ III, _Hinf_ I
(enzyme à quatre bases), _Bci_ I, _Bgl_ II,
_EcoRI, EcoRV, Hinc II, Hind III, Nde I et
_Spe_ I (enzyme à six bases). Les fragments
de restriction ont été séparés sur un gel à
1 % d'agarose et transférés par Southern-
blotting sur une membrane de nylon. Le pro-
duit d'amplification par PCR d'ADNmt d _A
cerana japonica_ (entre l'ARN_Leu_ de trans-
fert et la région CO II) a été marqué et utilisé
comme sonde pour l'hybridation. Les frag-
ments hybridés ont été visualisés et la
différenciation a été analysée à partir des
profils des bandes. Les données ont été
organisées en une matrice 1-0 et analy-
sées. Les distances euclidiennes ont été
calculées et un phénogramme construit à
l'aide d’UPGMA. On a obtenu de nombreux
profils RFLP (tableau I). Les échantillons
d' _A cerana_ provenant des différentes loca-
lités ont pu être séparés en six groupes comprenant : i) le Japon, ii) le Népal, le Viê-
tnam et la Thaïlande du Nord et du Centre,
iii) la Corée et Tsushima, iv) Taiwan, v) le
sud de la Thaïlande et vi) les Philippines
(fig 3). Ces résultats concordent avec les
données morphométriques de Ruttner

Apis cerana / ADNmt / variabilité / RFLP

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