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Original article

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

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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 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, Myanmer, 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-

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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.

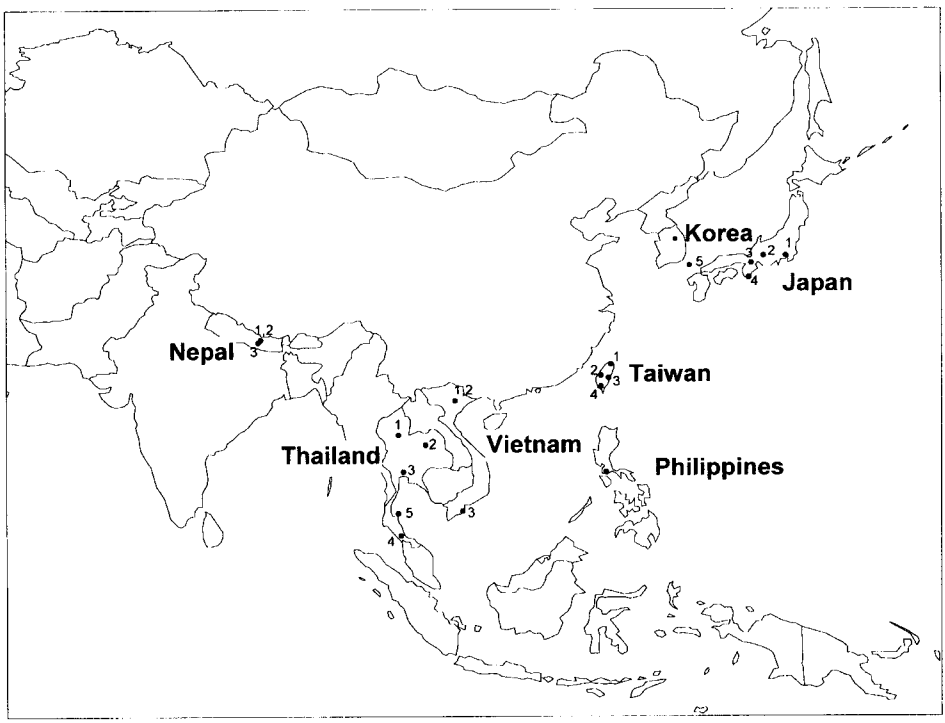

Fig 1. Locations of *A cerana* for mtDNA analysis. Sites: Japan: 1- Machida, Tokyo (2), 2) Iida, Nagano, 3) Uji, Kyoto,4) Koza, Wakayama, 5) Tsushima, Nagasaki (2); Korea: Yengweul, Kangwon-do; Taiwan: 1) Taipei, 2) Nantou, 3) Hualien, 4) Kaohsiung; Philippines: Lipa, Batangas; Vietnam: 1) Hanoi-A, 2) Hanoi-B (2), 3) My tho (2); Thailand: 1) Uttaradit, 2) Khon Kaen, 3) Bangkok, 4) Hatyai, 5) Samui; Nepal: 1) Kathmandu, 2) Lalitpur (2), 3) Chobal. Numbers in parentheses: number of colonies if > 1/site.

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, *EcoR* I, *EcoR* V, *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 Schuelf) (Maniatis et al, 1982) and hybridized with a labeled probe. The probe consisted of PCR-amplified product obtained from the use of two primers, E_2 and H_2 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 $tRNA^{Leu}$ 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 *EcoR* V 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 *EcoR* I, *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-

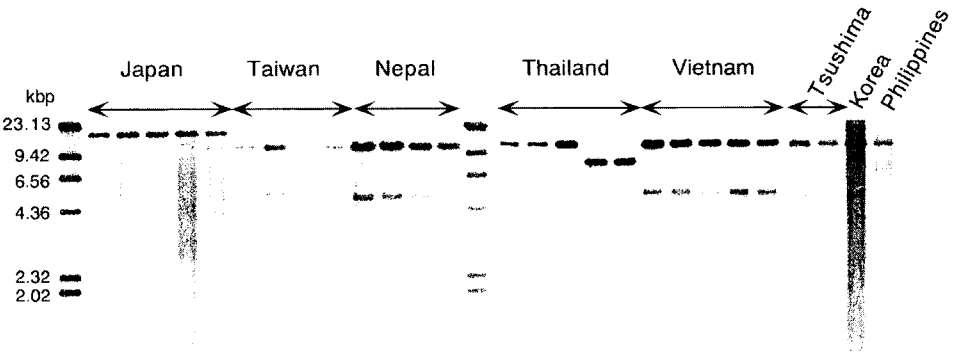

Fig 2. Fragments of mtDNA of *A cerana* after digestion with *EcoR* V and hybridized with labeled probe from $tRNA^{Leu}$ to CO II region.

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

Enzymes	*No of bands*	*No of patterns*	*Notes*
Hae III	10	10	Honshu group could be separated, high level of variation in Taiwan and Thailand
Hinf I	10	10	Honshu, Nepal, Thailand and Vietnam bees in group while others could be separated
Bcl I	6	4	
Bgl I	6	5	Variation within Taiwanese bees found
EcoR I	6	5	Korea-Tsushima group, Taiwan group, and Philippines distinguished from each other
EcoR V	5	3	Honshu group and south Thailand group could be separated
Hinc II	2	2	Philippines could be separated
Hind III	3	3	South Thailand group could be separated
Nde I	3	3	
Spe I	2	2	Samples from Philippines, Korea-Tsushima showed same patterns but different from others groups

ples. 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, *Eco*R 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-Tsushima 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-

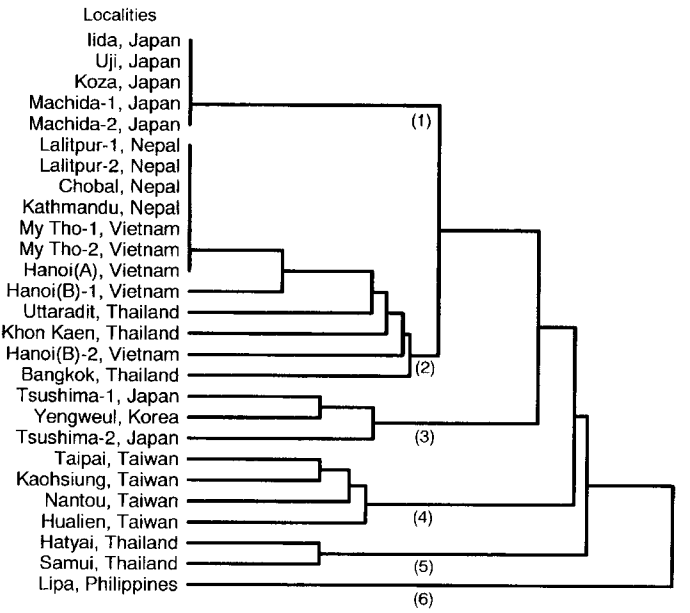

Fig 3. Cluster analysis of *A cerana* populations by UPGMA. Numbers in parentheses are groups which could be separated.

metric data of Ruttner (1988) and Limbipichai (1990).

These data indicate that using a probe from the intergenic region of *A mellifera* mtDNA from $tRNA^{Leu}$ 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 *EcoR* V 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.

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Zusammenfassung — Variation der mtDNA zwischen den Unterarten von *Apis cerana* ermittelt am Längenpolymorphismus von Restriktionsfragmenten. *Apis cerana* wurde von Ruttner (1988) auf Grund von morphometrischen Daten und der geographischen Verteilung in 4 Unterarten eingeteilt. Die Analyse der mtDNA-

Variation von Smith (1991, 1993) ergab, daß *A c japonica* den *Apis cerana* Gruppen des südostasiatischen Festlande sehr ähnlich ist. Sie schlug deshalb 3 Unterarten vor. Von 27 *A cerana* Völkern wurden jeweils 20-30 adulte Honigbienen an 22 verschiedenen Orten gesammelt (Abb 1). Die Proben wurden in absolutem Alkohol konserviert oder sofort mit Trockeneis oder flüssigem Stickstoff eingefroren und bei –70 °C für die spätere Analyse aufbewahrt. DNA von jeder Probe wurde mit 10 Restriktionsenzymen verdaut (*Hae* III, *Hinf* I (ein vier – Basen – Enzym), BclI, BglI, *EcoR* I, *EcoR* V, *Hinc* II, *Hind* III, *Nde* I and *Spe* I (ein sechs – Basen – Enzym). Die Restriktionsfragmente wurden in einem 1% Agarose Gel aufgetrennt und durch Southern-Blotting auf eine Nylonmembran übertragen. Das durch PCR vervielfältigte 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 Elemente wurden sichtbar gemacht und die Unterschiede im Bandenmuster analysiert. Die Daten wurden in einer 1-0 Matrix geordnet und analysiert. Die euklidischen Distanzen wurden berechnet und ein Phenogramm wurde mit Hilfe von UPGMA erstellt. Es wurden zahlreiche RFLP Muster erhalten (Abb 1). Wir konnten *A cerana* von unterschiedlichen Orten in 6 Gruppen ordnen: (1) eine japanische Gruppe; (2) eine Gruppe in Nepal, Vietnam und Nord-Zentral-Thailand; (3) eine Taiwan Gruppe, (4) eine Süd-Thailand Gruppe und (6) eine Gruppe in den Philippinen (Abb 3). Diese Befunde stehen im Einklang mit den morphometrischen Daten von Ruttner (1988) und Limbipichal (1990). Die größere Ähnlickeit von *A cerana* in Tsushima mit koreanischen als mit japanischen Honigibiene ist interessant. Jedoch werden mehr Proben, die das gesamte Verbreitungsgebiet der *A cerana* abdeckt, benötigt, bevor man über die Verwandtschaft dieser Population Aussagen machen kann.

***Apis cerana* / mtDNA / variabilität / RFLP**

Résumé — Étude de la variation de l'ADNmt chez les sous-espèces d'*Apis cerana* à l'aide du polymorphisme de longueur des fragments de restriction (RFLP). *A cerana* a été séparé par Ruttner (1988) en quatre sous-espèces en fonction de la morphométrie et de la répartition géographique. L'analyse par Smith (1991, 1993) de la variation de l'ADNmt a montré que les génomes mitochondriaux d'*A cerana* japonica 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 en 22 localités différentes (fig 1). Les échantillons ont été immergés dans de l'éthanol absolu ou immédiatement congelés dans de la carboglace ou de l'azote liquide et stockés à –70 °C pour les analyses ultérieures d'ADNmt. L'ADN de chaque échantillon a été digéré par dix enzymes de restriction : *Hae* III, *Hinf* I (enzyme à quatre bases), *Bcl* I, *Bgl* II, *EcoR* I, *EcoR* V, *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 transferrés par Southernblotting sur une membrane de nylon. Le produit d'amplification par PCR d'ADNmt d'*A cerana japonica* (entre l'ARN^{Leu} de transfert et la région CO II) a été marqué et utilisé comme sonde pour l'hybridation. Les fragments 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 analysées. 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 localités ont pu être séparés en six groupes comprenant : i) le Japon, ii) le Népal, le Viêtnam et la Thaïlande du Nord et du Centre, iii) la Corée et Tsushima, iv) Taïwan, v) le sud de la Thaïlande et vi) les Philippines (fig 3). Ces résultats concordent avec les données morphométriques de Ruttner

(1988) et de Limbipichai (1990). Il est intéressant de noter la ressemblance d'*A cerana* de Tsushima avec les abeilles coréennes plutôt qu'avec les japonaises. Il est néanmoins nécessaire d'analyser un plus grand nombre d'échantillons couvrant une plus grande partie de l'aire de répartition d'*A cerana* avant de conclure sur les relations entre ces populations.

***Apis cerana* / ADNmt / variabilité / RFLP**

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