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Rapid communication

Asymmetrical distribution of a mitochondrial DNA polymorphism between 2 introgressing honey bee subspecies

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Summary — Mitochondrial DNA from 62 samples of *Apis mellifera carnica* and *A m ligustica* collected from within areas of endemism and an area of known introgression was extracted and digested with the restriction enzymes *Acc*I, *Bcl*I, *Bgl*II, *Eco*RI and *Xba*I. Digestion with *Xba*I revealed a polymorphism with an asymmetrical distribution between the subspecies. This polymorphism occurs primarily in Austrian and Slovenian *A m carnica* and may be useful for studying introgression between this race and *A m ligustica*.

***Apis mellifera* / mitochondrial DNA / subspecies / population genetics**

INTRODUCTION

A number of authors have used restriction site polymorphism within the mitochondrial DNA of *Apis mellifera* to investigate the population genetics of this insect (Hall and Muralidharan, 1989, Smith *et al,* 1989, 1991; Rinderer *et al,* 1991, Sheppard *et al,* 1991a,b; Moritz and Meusel 1992). Comparisons in these studies were based in part on restriction site variation that differentiates the mitochondria of African and European honey bee subspecies. Unfortunately, few such "diagnostic" restriction site polymorphisms are known to occur among most honey bee subspecies, although size polymorphism may be relatively common (Moritz *et al,* 1986; Smith 1988; Arias *et al,* 1990; Cornuet *et al,* 1991; Garnery *et al,* 1992). Restriction site polymorphisms have been reported within particular subspecies (Smith and Brown 1990), although sample size limitations prevent estimation of their frequency or distribution.

In this paper we present evidence for the asymmetrical distribution of a mtDNA restriction site polymorphism between 2 honey bee subspecies, *Apis mellifera carnica* and *A m ligustica*, from within their areas of endemism. The distribution of the polymorphism suggests that it occurs commonly in *A m carnica,* and thus may be useful to study the introgression of this race with subspecies where the variant is in low frequency.

MATERIAL AND METHODS

Sixty-two colonies of honey bees (*A m carnica* and *A m ligustica* and natural hybrids of both) were sampled from apiaries stocked with local bees in Austria (2 apiaries, 12 samples), Slovenia (5 apiaries, 30 samples) and Italy (Reggio Emilia, 1 apiary, 10 samples and Udine, 1 apiary, 10 samples) (fig 1). Whereas south-eastern Austria and Slovenia are areas with naturally-occurring *A m carnica* and most of Italy contains endemic *A m ligustica*, northeastern Italy (Udine) is an area of known hybridization between both races (Nazzi, 1992). Adult worker bees were taken from brood combs and stored in liquid nitrogen. Subspecies identification consisted of analysis of 15 workers per sample for 36 morphological characters using discriminant analysis procedures modified from Ruttner *et al* (1978).

Total nucleic extraction of 2 bees per colony was performed by a modified phenolic extraction method (Sheppard and McPheron, 1991). The nucleic acid extracts were stored at -20°C in 10 mM Tris, 0.1 mM EDTA. The DNA was digested with the following restriction endonucleases according to the manufacturer's instructions (Bethesda Research Laboratories, Bethesda, MD USA): *Acc*I, *Bcl*I, *Bgl*II, *Eco*RI and *Xba*I. The digests were electrophoresed in 1% agarose gels, stained with ethidium bromide and photographed with UV-illumination. Transfer to nitrocellulose filters was carried out according to the method of Southern (1975). The filters were prehybridized and hybridized at 50°C using stan-

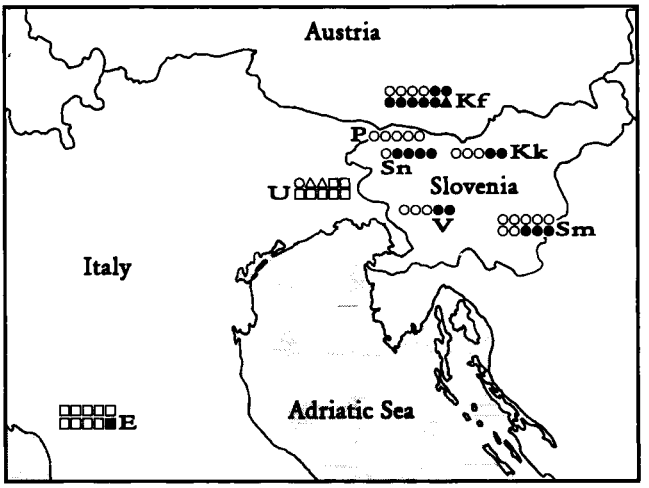

Fig 1. Morphological classification and *Xba*I restriction pattern distribution of sampled honey bee colonies. Each symbol represents one colony. Sampling locations are designated as follows: Austria: Klagenfurt (Kf) (2 apiaries); Slovenia: Podkoren (P), Senicno (Sn), Vhrnika (V), Kamnik (Kk) Semic (Sm) (2 apiaries); Italy: Udine (U) (Istituto Difesa delle Piante): Reggio Emilia (E) (Istituto Nazionale di Apicoltura). Symbols are assigned to the results of the morphometric analysis as follows: squares: *A m ligustica* morphology; triangles: intermediate morphology; circles: *A m carnica* morphology. Open symbols indicate *Xba*I restriction pattern 1, filled symbols indicate *Xba*I restriction pattern 2 (see text).

dard solutions containing salmon sperm DNA and 25% formamide (Maniatis *et al*, 1982; Sheppard *et al*, 1991b). Radioactive probe was produced through nick-translation of purified honey bee mitochondrial DNA (Sheppard *et al*, 1991b). Mitochondrial DNA restriction fragments were visualized by autoradiography.

RESULTS AND DISCUSSION

Discriminant analysis identified 42 of the colonies as *A m carnica* and 16 as A m ligustica when compared to published subspecies morphometric data (Ruttner 1988) (fig 2). Within the *carnica* group there is a detectable subdivision into 2 morphotypes corresponding to "Alpine" and "Pannonic" types of *A m carnica* (Ruttner, 1988). Three colonies had intermediate discriminant scores and were considered as hybrids. The geographic distribution of the 2 subspecies and their hybrids corresponds to published data (Ruttner, 1988; Nazzi, 1992).

The fragment sizes and patterns of all samples obtained by digestion with *Acc*I, *Bcl*I, *Bgl*II and *Eco*RI correspond to the restriction maps published by Smith (1988) for *A m ligustica* and *A m carnica* and to those of Arias *et al* (1990) for *A m ligustica*. For these enzymes, no variation was detected within or between the races.

Digestion with *Xba*I, however, produced 2 distinct restriction patterns differing by the presence of 1 cleavage site (fig 3). Pattern 2 consisted of four restriction fragments in our gels and appears to correspond to the Car2 pattern reported by Smith and Brown (1990) in their study of restriction site and length polymorphism in *A m carnica*. Pattern 1 consisted of three restriction site fragments, and with the enzymes in our study could not be unambiguously assigned to their named restriction morphs. The restriction site responsible for the difference between the patterns appears to correspond to the *Xba*I site q re-

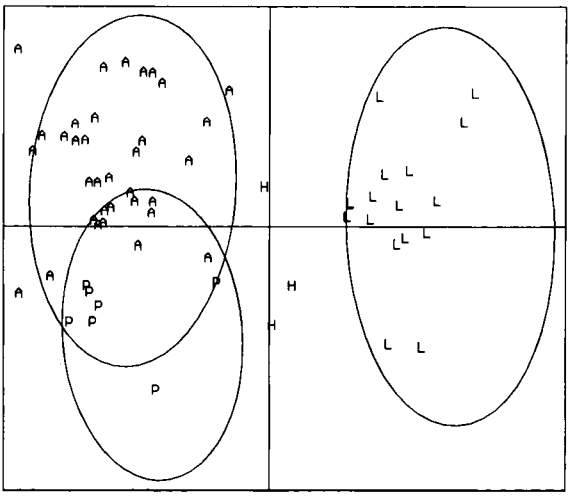

Fig 2. Positions of the samples investigated in a discriminant analysis. Abscissa: discriminant factor 1, ordinate: discriminant factor 2. The confidence curves (95%) are given. The discriminant factors and confidence curves were obtained from classified samples of honey bees from the Morphometric Data Collection in Oberursel. L: *A m ligustica*; A: *A m carnica* ("Alpine"); P: *A m carnica* ("Pannonic"); H: samples with intermediate discriminant scores, regarded as *carnica/ligustica* hybrids.

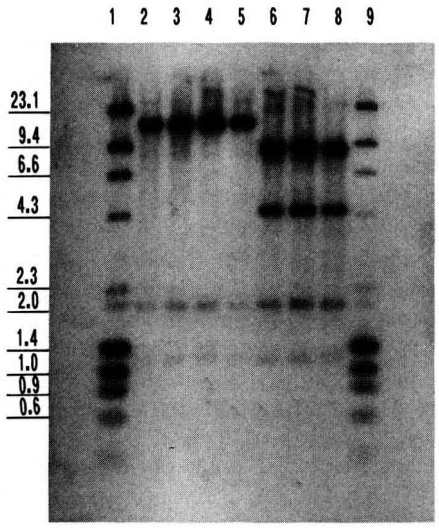

Fig 3. Autoradiograph of honey bee mitochondrial DNA showing the 2 *Xba*I patterns observed in this study. Lanes 1 and 9: lambda DNA digested with *Hin*dIII and *Phi*X DNA digested with *Hae*III. *Xba*I pattern 1: samples from Reggio Emilia (*A m ligustica*, lanes 2 and 5), Semic (*A m carnica*, 3) and Kamnik (*A m carnica*, 4), approximate fragment sizes estimated from the autoradiograph are 13.6, 1.95 and 1.3 kb. *Xba*I pattern 2: *A m carnica* samples from Klagenfurt (6, 7) and Senicno (8), approximate fragments sizes are 9.1, 4.4, 1.95 and 1.3 kb.

ported by Garnery *et al* (1992), with the presence of q producing pattern 2.

Of the 62 colonies examined, 42 exhibited pattern 1 and 20 pattern 2. In our samples, pattern 2 was predominantly limited to *A m carnica* – 19 out of 42 colonies from Austria and Slovenia showed this pattern. The distribution of the patterns is shown in figure 1.

Although our samples represent a relatively small portion of the geographic ranges of the 2 subspecies and the area of hybridization between them, pattern 2 was common in samples with *A m carnica* morphology and was found only once in *A m ligustica* and *ligustica/carnica* hybrids, respectively. Pattern 2 was widespread throughout the area of *A m carnica* sampled with no indication of a clinal distribution, but additional samples covering the total range of *A m carnica* would be needed to verify this. These results are in agreement with those of Garnery *et al* (1992), who found restriction site q to be present in 4 out of 6 *A m carnica* colonies and absent from all 9 *A m ligustica* colonies they sampled. If additional analyses of endemic *A m ligustica* and *A m carnica* confirm an asymmetrical distribution of the patterns, then this polymorphism may be useful to study genetic introgression between these subspecies.

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Résumé — Distribution asymétrique du polymorphisme de l'ADN mitochondrial entre deux sous-espèces d'abeilles domestiques (*Apis mellifera* L) introgressives. Soixante deux échantillons d'abeilles (*Apis mellifera carnica*, *A m ligustica* et leurs hybrides naturels) ont été prélevés dans les régions où elles sont endémiques et dans une région d'introgression connue (fig 1). L'identification des sous-espèces a été faite à partir de 15 ouvrières par échantillon par des analyses discriminantes portant sur 36 caractères morphologi-

ques. Après comparaison avec les données des sous-espèces publiées par Ruttner (1988) (fig 2), 42 colonies ont été classées comme carnica et 16 comme ligustica. Trois colonies ont obtenu une note discriminante intermédiaire et ont été considérées comme hybrides. Une extraction totale des acides nucléiques de 2 abeilles par échantillon a été réalisée (Sheppard et McPheron, 1991) et l'ADN a été digéré par les endonucléases de restriction *Acc* I, *Bcl* I, *Bgl* II, *Eco*R I et *Xba* I. Après électrophorèse sur gels d'agarose à 1%, l'ADN a été transféré sur une membrane de nitrocellulose et hybridé à 50 °C à l'aide de solutions standard contenant 25% de formamide (Maniatis *et al,* 1982; Sheppard *et al,* 1991b). La sonde utilisée a été de l'ADNmt purifié d'abeille marqué radioactivement par translation d'entaille. Les fragments de restriction de l'ADNm ont été visualisés par autoradiographie.

Alors que la digestion par *Acc* I, *Bcl* I, *Bgl* II et *Eco*R I n'a pas révélé de variation dans nos échantillons, la digestion par *Xba* I a produit 2 profils distincts se différenciant par la présence d'un seul site de clivage (fig 3). Le profil 2 était constitué de 4 fragments et semble correspondre au profil Car2 signalé par Smith et Brown (1990), tandis que le profil 1, constitué de 3 fragments, n'a pas pu être assigné de façon sûre à l'un des morphes de restriction. Le site de clivage responsable de la différence entre les 2 profils semble correspondre au site q signalé par Garnery *et al* (1992). Sur les 62 échantillons examinés, 42 ont présenté le profil 1 et 20 le profil 2. Le profil 2 est limité de manière prédominante à *A m carnica* – 19 des 42 colonies provenant d'Autriche et de Slovénie présentent ce profil (fig 1). Bien que nos échantillons ne représentent qu'une partie relativement restreinte de l'aire de répartition des 2 sous-espèces, le profil 2 est courant dans les échantillons qui présentent une morphologie de type *carnica* et rare dans les *ligustica* et les hybrides, mais il ne donne pas signe d'une distribution clinale. Si les analyses complémentaires d'abeilles *carnica* et *ligustica* endémiques confirment la distribution asymétrique des profils, il pourrait être utile d'utiliser ce polymorphisme pour étudier l'introgression génétique entre ces sous-espèces.

***Apis mellifera* / sous-espèce / ADN mitochondrial / génétique des populations**

Zusammenfassung — Asymmetrische Verteilung eines Polymorphismus der mitochondrialen DNA in zwei benachbarten geographischer Rassen der Honigbienen. 62 Proben von Honigbienen (Apis mellifera carnica, *A m ligustica* und natürliche Hybriden) wurden in Italien, Österreich und Slowenien gesammelt (Abb1).

Jeweils 15 Bienen eines Volkes wurden anhand von 36 Merkmalen nach Ruttner *et al* (1978) morphologisch klassifiziert. Eine Diskriminanzanalyse identifizierte 42 der Proben als *A m carnica* und 16 als *A m ligustica* (Abb 2). Drei Proben hatten intermediäre Diskrimanzwerte und wurden als Hybriden betrachtet.

Die DNA von jeweils 2 Bienen pro Probe wurde nach der Methode von Sheppard und McPheron (1991) extrahiert und mit den Restriktionsendonukleasen *Acc* I, *Bcl* I, *Bgl* II, *Eco*R I und *Xba* I abgebaut. Nach Elektrophorese in 1% Agarosegelen wurden die Fragmente auf Nitrocellulosefilter transferiert (Southern, 1975) und bei 50 °C unter Standardbedingungen hybridisiert (Maniatis *et al,* 1982; Sheppard *et al,* 1991b). Die radioaktive Sonde wurde durch Nick-Translation gereinigter mtDNA von Honigbienen hergestellt (Sheppard *et al,* 1991b). Die mitochondrialen Restriktionsfragmente wurden durch Autoradiographie sichtbar gemacht.

Während die durch den Abbau der mtDNA mit *Acc* I, *Bcl* I, *Bgl* II und *Eco*R I hergestellten Fragmentmuster in allen untersuchten Proben keine Variation zeigten, entstanden durch den Abbau mit *Xba* I zwei deutlich verschiedene Muster, die sich in einer Schnittstelle unterscheiden (Abb 3). Unser Muster 2 bestand aus 4 Fragmenten und scheint dem von Smith und Brown (1990) veröffentlichten Car2 Muster zu entsprechen, während Muster 1 keinem ihrer Fragmentmuster widerspruchsfrei zugeordnet werden konnte.

Die für den Unterschied zwischen den Fragmentmustern verantwortliche Schnittstelle scheint der von Garnery *et al* (1992) gefundenen Schnittstelle q zu entsprechen.

Von den 62 untersuchten Proben, zeigten 42 Muster 1 und 20 Muster 2. Muster 2 ist weitgehend auf *A m carnica* beschränkt – es ist in 19 von den 42 in Österreich und Slowenien gesammelten Proben vorhanden (Abb1). Obwohl unsere Sammlung nur einen geringen Anteil der Verbreitungsgebiete beider Unterarten repräsentiert, war Muster 2 in Proben mit *A m carnica* Morphologie verbreitet und wurde in *A m ligustica* sowie Hybriden jeweils nur einmal gefunden. Aus den Proben unserer Sammlung ergibt sich jedoch kein Hinweis auf eine klinale Verbreitung. Wenn sich die asymmetrische Verteilung dieser Fragmentmuster durch weitere Analysen von endemischer *A m carnica* und ligustica bestätigen läßt, könnte dieser Polymorphismus zur Untersuchung der genetischen Introgression zwischen diesen beiden Subspezies genutzt werden.

***Apis mellifera* / mtDNA / Unterart / Genetik der Populationen**

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