Mitochondrial DNA sequence data provides further evidence that the honeybees of Kangaroo Island, Australia are of hybrid origin

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Summary — Morphological, multivariate and allozyme data show that the honeybee populations of Kangaroo Island, Australia, are more similar to *Apis mellifera ligustica* than *A m mellifera*. However, our sequence analysis of the ATPase 6, COIII, cytochrome b and ND2 mitochondrial genes shows a significant association, 100% according to bootstrap resampling, between the Kangaroo Island haplotype and *A m mellifera*. Therefore it is likely that the Kangaroo Island population was originally established from hybrids. We conclude that the ancestral populations of *A m mellifera* contained both the *'mellifera'* haplotypes reported here, with complementary fixations in Tasmania and on Kangaroo Island. Since *A m mellifera* mtDNA haplotypes are shared between Australian honeybees classified as *A m mellifera* and *A m ligustica*, then the dichotomous nature of mtDNA lineages cannot be used to identify bees to subspecies in Australia.

***Apis mellifera* / mitochondrial DNA / phylogenetic analysis / Kangaroo Island / Australia**

INTRODUCTION

All accounts (Hopkins, 1886; Eckert, 1958; Woodward, 1993) agree that the founding *Apis mellifera ligustica* population of Kangaroo Island, Australia, had the opportunity to hybridise with *A m mellifera* on mainland Australia before being transferred to the island. Therefore, although subsequent importations were made directly from Naples (Eckert, 1958), it could well be that this population is not 'pure' *A m ligustica* as generally thought.

Ruttner (1976) made a morphological comparison between three colonies from Kangaroo Island and specimens collected in Italy, and concluded that the Kangaroo Island bees showed the 'typical morphological characters' of *A m ligustica* from Italy, except for wing shape and venation. The divergence of wing shape from that expected in *A m ligustica* was not attributed

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to hybridisation because '...hybrids always show deviations in several characters'. His conclusion was that this type developed on Kangaroo Island, substantiated by the high levels of intracolonial variation.

Oldroyd et al (1993), using multivariate analysis of morphological characters and allozymes, found that the Kangaroo Island populations were *A m ligustica*, but mtDNA restriction mapping suggested that they were of *A m mellifera* origin. Oldroyd et al (1993) concluded that the reference material was inadequate to determine the racial origin of the Kangaroo Island population, but that recent hybridisation had not occurred.

We now provide a molecular comparison of three populations of honeybees present in Australia using mitochondrial DNA (mtDNA). All reference material was amplified for the size polymorphic COI/COII region, and restriction digests of the ND1/ND4 region were checked against published restriction maps because of the difficulty in locating reliable reference populations of certain subspecies status in Australia (classifications are usually based on the judgement of apiarists). Sequences were obtained from the ATPase 6, COIII, cytochrome b and ND2 genes. We compared colonies from Kangaroo Island with *A m mellifera, A m ligustica* and *A m scutellata* to determine their relationship to each other.

MATERIALS AND METHODS

For this study we amplified and restricted or sequenced mtDNA from one worker from each colony of four managed Kangaroo Island colonies, three feral Kangaroo Island colonies, two *A m ligustica* colonies, two *A m mellifera* colonies and one *A m scutellata* colony. Kangaroo Island specimens were supplied by D Paton. *A m ligustica*-derived specimens were supplied by R Stephens Apiaries, Tasmania. *A m mellifera* specimens were collected by SK and B Oldroyd in Tasmania. *A m scutellata* was supplied in alcohol by H Glenn Hall (University of Florida). Voucher specimen identity codes (IC), date received, supplier and locality of colonies sequenced are shown in table I.

A m mellifera and *A m ligustica* reference specimens were sent to the US Department of Agriculture in Baton Rouge for identification (for methods see Oldroyd et al, 1995).

Table I. Voucher specimen identity codes (IC), date received, supplier and locality of colonies sequenced.

Bees	*Identity code*	*Date*	*Source*	*Locality*
Kangaroo Island	ABZO	13/12/90	D Paton	Kangaroo Island, South Australia
A m scutellata	ABZR (VCN7A in the Hall collection)	27/6/90	HG Hall	United States
A m mellifera	Tas 81, 86	9/3/93	BP Oldroyd, and SK	Tarraleah, Tasmania
A m ligustica	Tas 49, 52	9/3/93	R Stephens Apiaries	Mole Creek, Tasmania

Total DNA was extracted from single bees by modification of standard procedures as in Crozier et al (1991).

The ATPase 6/COIII, cytochrome b, ND2 and COI/COII regions were amplified using primers (table II) designed from the known complete sequence (Crozier and Crozier, 1993) and Taq polymerase (United States Biochemical Corporation) in a Perkin Elmer Cetus thermal cycler for 35 cycles under the following conditions: denaturation at 92 °C for 1 min, annealing at 53 °C for 1 min, extension at 70 °C for 1 min. The ND1/ND4 region was amplified for 30 cycles under the following conditions: denaturation at 92 °C for 1.5 min, annealing at 50 °C for 1 min, extension at 70 °C for 5 min. All products were purified with Millipore Ultrafree units.

The COI/COII PCR product was sized by electrophoresis on a 2% agarose gel against φX174 cut with *HaeIII*. The bands were visualised by ethidium bromide staining. The ND1/ND4 region was digested with *PvuII*, *SpeI* and *NdeI* as recommended by the supplier (Promega). The digested fragments were separated on a 2% agarose gel and visualised by ethidium bromide staining.

Direct sequencing of the ATPase 6/COIII, cytochrome b and ND2 gene regions from single-stranded PCR product was achieved by a modification of the protocol by Kessing et al (1989), as in Koulianos and Crozier (1991).

Sequence information was entered using the MacVector package (IBI). Phylogenetic analyses were carried out using the maximum likelihood method implemented using the program DNAML in the PHYLIP package (Felsenstein, 1993) and maximum parsimony using PAUP (Swofford, 1993). Maximum likelihood trees were obtained by searching for the best tree with global rearrangements. Parsimony trees were obtained by the branch and bound method. Support for the nodes was obtained by a minimum of 1 000 bootstrap replications. The transition/transversion ratio used for both methods was 3.9, as obtained by maximising the DNAML results.

RESULTS

No mitochondrial variation was found in the seven colonies from Kangaroo Island, ie, all Kangaroo Island colonies had the same length polymorphism for the COI/COII region, the same restriction digest profiles,

Table II. Primers used for amplification and sequencing.

Region	*Primers*	*Coordinates*
ATPase 6	CGATTATCAGCAAATTTAATTTCTG	5 070–5 094
COIII	GTATACCTTGAAATGTTCTTTCTCG	5 468–5 492
Cytochrome b	TATGTACTACCATGAGGACAAATATC	11 400–11 425
	ATTACACCTCCTAATTTATTAGGAAT	11 859–11 884
ND2	TCCACAAATAAAACCCCAAGATT	649–671
	CTAAAATTAATTCATGAAC	923–941
	GAAGTGTAAGTGCAAAATATCTATG	1 435–1 459
COI/COII	TCTATACCACGACGTTATTC	3 089–3 109
	GATCAATATCATTGATGACC	3 917–3 937
ND1/ND4	GTAGCATTTTTAACTTTATTAGAACG	13 142–13 168
	GGAGCTTCAACATGAGCTTT	9 335–9 364

Primers were designed from the published honeybee sequence in Crozier and Crozier (1993) and are listed 5' to 3'. Coordinates as in Crozier and Crozier (1993).

and the same mtDNA sequence for the ATPase 6/COIII, cytochrome b and ND2 regions.

All reference populations show size classes that are consistent with that subspecies (table III). These size classes result from different combinations of two sequence fragments named P and Q in Cornuet et al (1991). The Q sequence is observed in our *A m ligustica* reference population. It is also found in all other northern Mediterranean species (Garnery et al, 1992). The other sizes (PQ, PQQ and PQQQ) are known in *A m mellifera* and several African races (Garnery et al, 1992). Therefore we can distinguish *A m mellifera* and African haplotypes from those of the northern Mediterranean species. Here the size classes PQQ (present in the Kangaroo Island colonies) and PQQQ (present in the *A m mellifera* reference population) are compatible with *A m mellifera,* but not with *A m ligustica*.

The presence or absence of *BglII, HindIII, PvuII, SpeI* and *NdeI* restriction sites are also shown in table III. All reference populations show restriction patterns typical of their subspecies except the population from Tarraleah, Tasmania, which differs from published *A m mellifera* maps (Garnery et al, 1992) by one site. Tarraleah bees were described by Ruttner (1976) as true *A m mellifera* on morphological grounds; our samples were sent to the US Department of Agriculture in Baton Rouge for identification and they too were found to be morphologically *A m mellifera* (for results see Oldroyd et al, 1995). The Kangaroo Island population differs here from *A m ligustica* and *A m mellifera* by one and four sites respectively, but from published restriction maps (Garnery et al, 1992) by one and two sites respectively.

Sequences (fig 1) were obtained from samples of 12 colonies: seven Kangaroo Island colonies, two *A m mellifera* colonies, two *A m ligustica* colonies and one *A m scutellata* colony. That from our *A m ligustica* reference population differed by three substitutions to that in Crozier and Crozier (1993).

Based on the sequence data, the mean divergence between the mtDNA haplotypes as estimated by Kimura's (1980) method was calculated. Contrary to the restriction digest profiles, from the distances it is clear that the Kangaroo Island haplotype is closer to *A m mellifera* ($d = 0.0072$) than to *A m ligustica* ($d = 0.0139$). The largest value

Table III. Restriction site and length polymorphisms for the Australian haplotypes and *A m scutellata*.

Sample	BglI *(b)*	HindIII *(f)*	PvuII *(a)*	SpeI *(c)*	SpeI *(t)*	NdeI *(u)*	NdeI *(v)*	*Length polymorphism*
Australian *'ligustica'*	+	+	+	–	–	+	+	Q
Kangaroo Island	+	+	+	–	–	+	–	PQQ
Australian *'mellifera'*	+	–	+	+	+	–	–	PQQQ
A m scutellata	–	+	+	+	–	–	–	PQ

Presence of a restriction site is indicated by + and the absence by –. Presence/absence of *PvuII*, *SpeI* and *NdeI* sites was obtained from restriction digests. Presence/absence of *BglII* and *HindIII* sites was obtained from the sequences. Corresponding restriction sites of Garnery et al (1992) are shown in brackets. The length polymorphisms result from different combinations of two sequence fragments named P and Q in Cornuet et al (1991).

obtained was that between *A m mellifera* and *A m scutellata* ($d = 0.0220$).

Both maximum likelihood and bootstrapped parsimony analysis unequivocally link the Kangaroo Island bees with *A m mellifera* (fig 2).

DISCUSSION

Comparison of the restriction map of Oldroyd et al (1993) to those of Smith (1988), Smith and Brown (1990), and Cornuet and Garnery (1991) does not unequivocally support the suggestion that the Kangaroo Island mtDNA haplotype derives from *A m mellifera*. While the *EcoRI* and *BclI* restriction patterns of Kangaroo Island bees are closer to that of *A m mellifera* than to that of *A m ligustica*, the *HindIII* pattern links them to *A m ligustica* and the *XbaI* pattern could be interpreted as linking them to either. Also, from our restriction data, the Kangaroo Island population differs from *A m ligustica* by only one site compared to four from *A m mellifera*. To the contrary, our sequence data show that the Kangaroo Island haplotype is more similar to that of Tasmanian *A m mellifera*. However, it still differs from it by 11 base substitutions.

Because the Kangaroo Island population resembles *A m ligustica* morphologically (Ruttner, 1976; Oldroyd et al, 1993), but has *A m mellifera* mitochondria (we suspect that the sequence data are more informative), then it is most likely that it was established from hybrids. Liguria, from which the colonies are reputed to have originated, is a natural hybrid zone for *A m mellifera* and *A m ligustica* (Badino et al, 1982; Cornuet, 1983). Badino et al (1982) found a gradient of MDH allelic frequencies clearly indicating that *A m mellifera* genes are found as far east as Genoa. Although no mtDNA data are available from this particular region, it can be expected that *A m mellifera* mtDNA can also be found in Genoa, regardless of the bees morphometric similarity to *A m ligustica* (Ruttner, 1988). It is possible that the *'mellifera'* haplotype would be maintained if *A m mellifera* had a selective advantage, regardless of subsequent importations from Naples. The fixation of such haplotypes has been simulated by Moritz and Meusel (1992).

The possibility of recent illegal importations seems remote because no mitochondrial variation was found among any of the island colonies. We are therefore left with the following possibilities.

First, the ancestral populations of *A m mellifera* contain both the *'mellifera'* haplotypes we report here, with complementary fixations of the two haplotypes in Tasmania and on Kangaroo Island. This possibility is supported by the sequence data and the size of the COI/COII intergenic region.

Second, the sequence differences between the Kangaroo Island and Tasmanian *A m mellifera* bees arose following their establishment which would imply an implausibly high rate of sequence evolution; 5×10^6% per Myr compared to 2% per Myr for mammals (Brown et al, 1979) and *Drosophila* (DeSalle et al, 1987; Monnerot et al, 1991).

Since *A m mellifera* mtDNA haplotypes are shared between Australian honeybees classified as *A m mellifera* and *A m ligustica*, then the dichotomous nature of mtDNA lineages cannot be used to identify bees to subspecies in Australia, although frequency differences could still be used to characterise populations. This situation may be encountered in all areas where bees have been imported. Also, these findings emphasise the difficulty of locating reliable reference populations of certain subspecies' status in Australia. Such uncertainty is perhaps to be expected when the occurrences of a social insect are determined not only by natural processes but also by human activities, planned and unplanned.

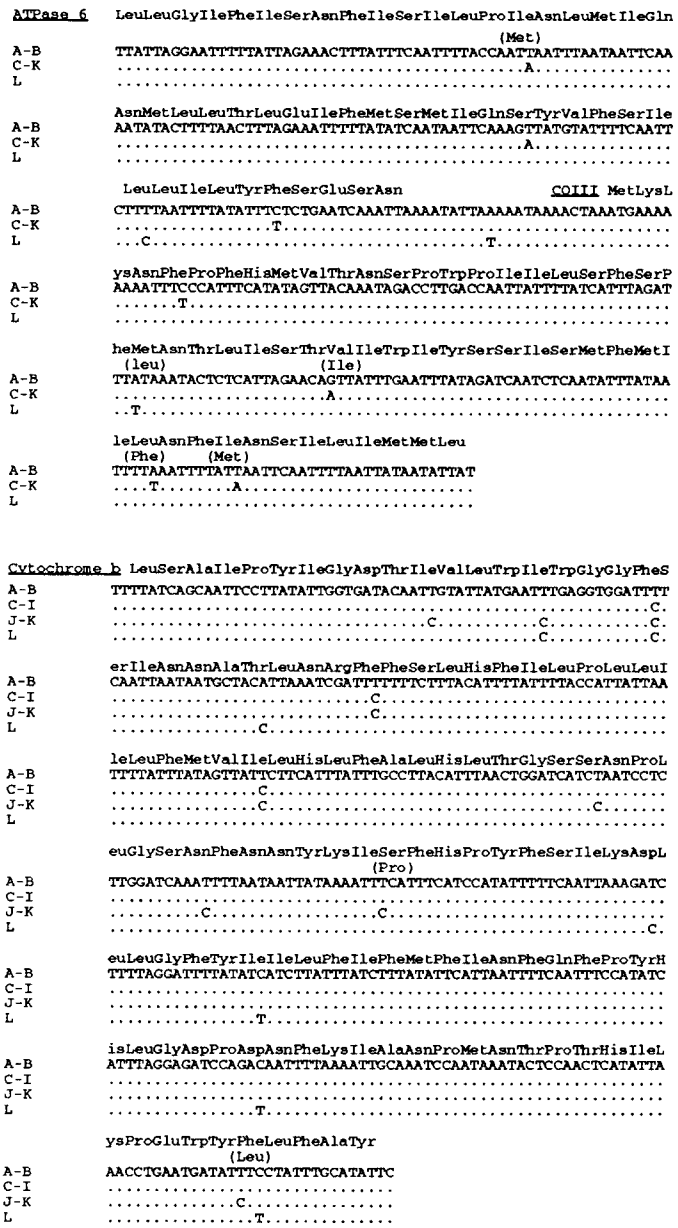
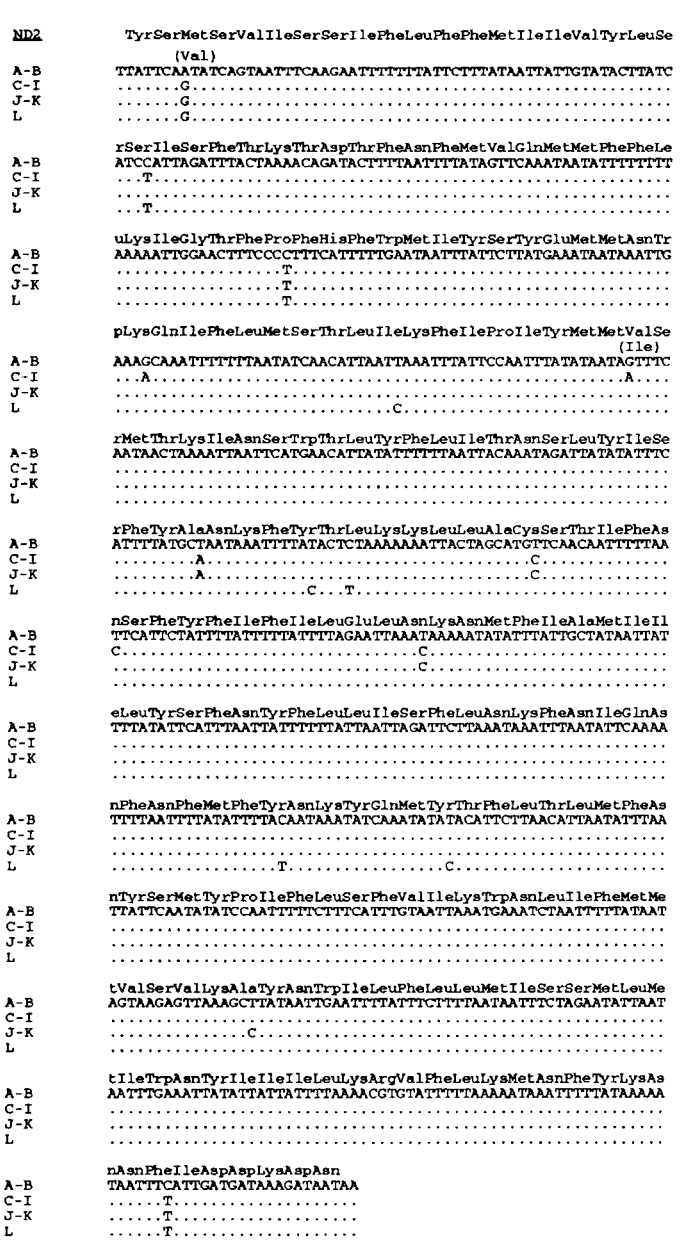


Fig 1. Nucleotide sequences obtained from the ATPase 6, COIII, cytochrome b and ND2 mitochondrial genes (1 477 bps in length). The amino acids correspond to the codon directly below in A. The amino acids resulting from the base substitutions are in brackets. Dots represent bases the same as in A.



(A and B are *A m ligustica*, C through F are managed hives from Kangaroo Island, G through I are feral hives from Kangaroo Island, J and K are *A m mellifera*, and L is *A m scutellata*.)

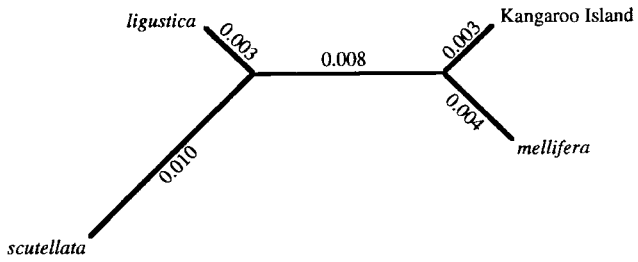


Fig 2. The relationship of the four haplotypes to each other. The tree and branch lengths shown are those obtained using maximum likelihood (Ln likelihood = −1 910.89). All branches are significantly positive. All 1 000 bootstrap replicates returned the same tree under parsimony.

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Zusammenfassung — MtDNA Sequenzdaten liefern weitere Hinweise für die Abstammung der Honigbienen von Hybriden auf den Känguruh-Inseln, Australien. Alle bisherigen Darstellungen (Hopkins, 1886; Eckert, 1958; Woodward, 1993) stimmen darin überein, daß die Gründerpopulationen von *A m ligustica* der Känguruh-Inseln Gelegenheit hatte, sich vor dem Transport auf die Inseln mit *A m mellifera* zu kreuzen. Obwohl alle folgenden Bieneneinfuhren direkt aus Neapel (Eckert, 1958) erfolgten, war damit die Möglichkeit gegeben, daß die Inselpopulation nicht, wie allgemein angenommen, eine reine *A m ligustica* darstellt. Morphologische (Ruttner, 1976), multivariate und isoenzym-Daten (Oldroyd et al, 1993) ergaben, daß die Population der Känguruh-Inseln mehr Ähnlichkeiten mit *A m ligustica* als mit *A m mellifera* aufweisen. Dagegen zeigen unsere Sequenzanalysen der mitochondrialen Gene für ATPase 6, COIII, Cytochrom b und ND2 eine signifikante Verbindung zwischen dem Känguruh-Inseln Haplotyp und der *A m mellifera* Vergleichsprobe aus Tasmanien. Bei der "bootstrap resampling" Methode ergab sich eine 100% Übereinstimmung (Abb 2). Aus diesem Grund ist es wahrscheinlich, daß die Population der Känguruh-Inseln ursprünglich aus Hybriden bestand. Die Wahrscheinlichkeit eines kürzlich erfolgten illegalen Imports scheint ausgeschlossen, denn es wurde keine mitochondriale Variation zwischen den Völkern der Insel gefunden. Daraus schließen wir, daß in der Urpopulation von *A m mellifera* beide hier beschriebenen *mellifera* Haplotypen verhanden waren, und daß sie sich komplementär in Tasmanien und auf den Känguruh-Inseln fixiert haben. Da die beiden *A m mellifera* mtDNA Haplotypen bei beiden als *A m mellifera* und *A m ligustica* klassifizierten australischen Honigbienentypen vorkommen, kann die zweigeteilte Natur der mtDNA Abstammungslinien nicht dazu benutzt werden, die Bienen in Australien bestimmten Rassen zuzuordnen. Diese Situation könnte in allen Gegenden vorkommen, in die Bienen importiert wurden. Außerdem zeigen die Ergebnisse deutlich die Schwierigkeiten, zuverlässige Bezugspopulationen mit einem bestimmten Rassestatus in Australien zu lokalisieren. Diese Unsicherheit ist wahrscheinlich immer zu erwarten, wenn das Vorkommen von sozialen Insekten nicht nur durch natürliche Vorgänge sondern auch durch geplante oder versehentliche menschliche Aktivitäten beeinflußt wird.

***Apis mellifera* / mitochondriale DNA / phylogenetische Analyse / Känguruh-Inseln / Australien**

Résumé — Les données de séquences de l'ADNmt fournissent une nouvelle preuve de l'origine hybride des abeilles mellifères (*Apis mellifera* L) de l'île de Kangaroo, Australie. Tous les rapports admettent que la première population d'*Apis mellifera ligustica* de l'île de Kangaroo a eu l'occasion de s'hybrider sur le continent australien avec *A m mellifera* avant son transfert sur l'île. Par conséquent, bien que les importations postérieures aient été faites directement de Naples (Eckert, 1958), il se pourrait que cette population ne soit pas pure *ligustica* comme on le pense généralement. Les analyses morphologiques (Ruttner, 1976), multivariées et allozymiques (Oldroyd et al, 1993) montrent que les populations de l'île de Kangaroo sont plus proches d'*A m ligustica* que d'*A mellifera*. Pourtant notre analyse de séquence de l'ATPase 6, du COIII, du cytochrome b et des gènes mitochondriaux ND2 montre une association significative, à 100 % selon la technique de «bootstrap» entre l'haplotype de Kangaroo et notre échantillon de référence *A m mellifera* provenant de Tasmanie (fig 2). Il est donc probable que la population d'origine de l'île Kangaroo s'est établie à partir d'hybrides. La possibilité d'importations récentes illégales semble peu probable car dans aucune des colonies de l'île on n'a trouvé de variation mitochondriale. Nous en concluons que les populations ancestrales d'*A m mellifera* renfermaient les deux haplotypes *«mellifera»* mentionnés ici, avec des fixations complémentaires en Tasmanie et sur l'île de Kangaroo. Puisque les abeilles australiennes classées comme *A m ligustica* et *A m mellifera* partagent les haplotypes d'ADNmt d'*A m mellifera*, la nature dichotomique des lignées d'ADNmt ne peut être utilisée pour identifier en Australie les abeilles au niveau de la sous-espèce. Cette situation peut se rencontrer dans toutes les régions où des abeilles ont été importées. Ces résultats soulignent aussi la difficulté de localiser en Australie des populations de référence fiables de certaines sous-espèces. Il faut peut-être s'attendre à cette incertitude lorsque la présence d'un insecte social est déterminée non seulement par les processus naturels mais aussi par les activités humaines, voulues ou non.

***Apis mellifera* / génétique population / DNA mitochondrial / phylogenèse / Australie**

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