Racial admixture of *Apis mellifera* in Tasmania, Australia: similarities and differences with natural hybrid zones in Europe

BENJAMIN P. OLDROYD†*, JEAN-MARIE CORNUET†‡, DARRYL ROWE†, THOMAS E. RINDERER§ & ROSS H. CROZIER†

†*School of Genetics and Human Variation, La Trobe University, Bundoora, Vic 3083, Australia, ‡Laboratoire de Neurobiologie Comparée des Invertébrés, INRA-CNRS URA 1190, La Guyonnerie, B.P. 23, 91440 Bures-sur-Yvette, France and §Honey-Bee Breeding, Genetics and Physiology Research Laboratory, USDA-ARS, 1157 Ben Hur Road, Baton Rouge, LA 70820, U.S.A.*

Feral and domesticated honey bees were collected across the island state of Tasmania, Australia, and typed for malate dehydrogenase and a mitochondrial DNA polymorphism. They were also compared morphometrically with reference specimens of *Apis mellifera ligustica* and *A. m. mellifera* from Europe. These measures were correlated with temperature and elevation. In warmer coastal regions, the two subspecies readily hybridize and most samples showed evidence of considerable hybridization. In cooler mountain regions, there is much less hybridization, with the *A. m. mellifera* subspecies characteristics strongly predominating. There is no evidence for cyto-nuclear incompatibilies between these subspecies or for clines caused by direct selection. We hypothesize that *A. m. ligustica* and F_1 hybrids have lower fitness than *A. m. mellifera* in cooler regions, and that there may be assortative mating in cooler regions only. The significance of these results for the understanding of honey bee hybrid zones in Europe is discussed.

Keywords: *Apis mellifera*, cline, hybrid zone, malate dehydrogenase, mitochondrial DNA, morphometrics.

Introduction

Understanding how two adjoining subspecies maintain their identity is of considerable interest because the phenomenon may represent an early stage of speciation (Barton & Hewitt, 1985). Two situations have to be considered: (i) when adjoining subspecies have evolved parapatrically, and (ii) when adjoining subspecies have diverged allopatrically but have become reunited by secondary contact. The first case is the normal consequence of genetic drift and selection resulting in genetically distinct populations. The second case is much more interesting as there is the potential for competition between two expanding subspecies which are already genetically distinct. In some cases this may lead to the displacement of one subspecies by the other, the merging of the two (Moore, 1977) or, if speciation has proceeded far enough, a hybrid zone may develop characterized by coincident clines for the

characters differentiating the two subspecies. Equally fit alleles may diffuse across the hybrid zone. The width of the cline will depend on how recently the contact was made and the rate of dispersal of individuals (Endler, 1977).

If the fitness in F_1 hybrids of the two subspecies differs from that of parents, a hybrid zone, or tension zone, may become virtually permanent, impeding gene flow between adjoining subspecies for many generations (Moore, 1977; Moore & Buchanan, 1985). The width of such a zone depends not only on the rate of dispersal, but also on the relative fitness of F_1s (Hewitt, 1988). Following Bazykin (1969) the model of Barton & Hewitt (1985) requires hybrid unfitness (from the breakdown of coadapted gene complexes or abnormalities in chromosomal pairing in F_1s) but no environmental gradient for the maintenance of hybrid zones. In contrast, Moore (1977) and Moore & Buchanan (1985) show that permanent hybrid zones can result from heterosis in F_1s, coupled with an environmental gradient in which F_1s are fitter than either parent within

*Correspondence.

the zone of intermediate ecology between the native ranges of the parental subspecies.

The western honey bee *Apis mellifera* is native to Europe, Africa and western Asia. This broad geographical distribution has resulted in the evolution of three distinct evolutionary branches: western European (M), northern Mediterranean (C) and African (A). These branches were initially discovered using morphometrics (Ruttner *et al.*, 1978; Ruttner, 1988) and have been largely confirmed by mitochondrial DNA studies (Smith, 1991; Garnery, 1992; Garnery *et al.*, 1992). As defined by morphometrics, these branches represent one to eight subspecies including *A. m. mellifera* (branch M) and *A. m. ligustica* (branch C). In northern Italy, these two subspecies have formed a contact zone which has been well characterized by allozyme and morphometric studies (Cornuet, 1983; Badino *et al.*, 1983, 1984, 1988a,b; Comparini & Biasiolo, 1991; Nazi, 1992; Meixner *et al.*, 1993). This contact zone provides an ideal system for studying a secondary contact between two formerly allopatric subspecies. Because of the high level of genetic divergence between *mellifera* and *ligustica*, there are morphological, mitochondrial and allozyme markers available to discriminate between them (reviewed by Oldroyd *et al.*, 1992). Secondly, these two subspecies have been exported to many places around the world during the past century, providing opportunities to study artificial contact zones in new environments.

Tasmania (Australia) is such a new environment. In 1831, a first *Apis mellifera* colony was introduced from Great Britain, presumably of the subspecies *mellifera*. By 1860, honey bees were apparently feral and widespread throughout the island (Ayton, 1977). The first reputedly 'Italian' (*A. m. ligustica*) bees were introduced to the island more than 50 years later in 1884 (H. Ayton, personal communication). Bees of Italian lineage are used extensively for commercial and amateur beekeeping. Several thousand *ligustica* queens are imported into the state each year from queen propagation farms in subtropical Queensland and New South Wales, and many more are produced locally. After so many years of extensive propagation of *ligustica*-type bees, one might expect the extinction of the *mellifera* type, unless strong selection or barriers to hybridization prevent gene flow between the two populations; and yet Ruttner (1976) reported a population of dark bees from Tarraleah, Tasmania, that are 'unhybridized' *A. m. mellifera*.

Here, we report on the genetic structure of the honey bee population of Tasmania, using morphometrics and malate dehydrogenase as nuclear markers, and mitochondrial DNA RFLPs as cytoplasmic markers. 'Pure' *A. m. ligustica* and *A. m. mellifera* are

generally identifiable by malate dehydrogenase (*Mdh*) allele frequencies (Cornuet, 1983) and mt-DNA RFLPs (Garnery, 1992). Studies which simultaneously examine nuclear and cytoplasmic markers (cytonuclear systems) provide the opportunity to obtain insight into the nature of the hybridization process (Arnold, 1993). As clines correlated with environmental temperature have been reported for morphometric characters (Ruttner, 1988; Daly *et al.*, 1991) and *Mdh* allele frequencies (Nielson *et al.*, 1994) we looked for significant correlations between genetic markers and environmental temperature. By these means, we attempt to determine if the observed clines are environmentally induced or are the result of a contact zone. In the light of these analyses, the coexistence of the two subspecies in Tasmania and in contact zones in Europe are discussed.

Materials and methods

Field collections

Collections were made from individual colonies across Tasmania (Fig. 1). Bees were caught outside colonies in sweep nets or by aspiration, or if the colony was in a moveable frame hive, by scraping bees off honey combs. Bees were killed immediately by freezing in liquid nitrogen. For each colony, about half the bees were preserved in liquid nitrogen and half in ethanol. Except for a collection from commercial colonies originating from Mole Creek, all bees were feral. Feral colonies were defined as those that met at least one of the following criteria: (i) colonies in tree hollows (nine colonies); (ii) recently hived swarms that had not been requeened (24 colonies); and (iii) colonies in neglected or abandoned apiaries where the owner or apiary inspector stated that there had been no queen replacement by the beekeeper for at least 10 years (63 colonies). Up to 12 colonies were sampled at any one site, but more usually three to seven (Fig. 1).

In addition to feral colonies, bees were sampled from 20 colonies at Tarraleah. The apiary was established in 1969 from feral bees from the surrounding eucalyptus forest by L. Slater (Ayton, 1977). Because Ruttner (1976) reported that the morphology of the bees of Tarraleah 'corresponds almost exactly to the original bee from north-west Europe', the area surrounding Mr Slater's apiary has been declared a sanctuary for the preservation of the bees which are thought to be an isolated population of *A. mellifera mellifera*. However, the sanctuary has little legal force and is not geographically isolated. Mr Slater does not undertake any selection or queen production. The bees interbreed freely with wild colonies which are
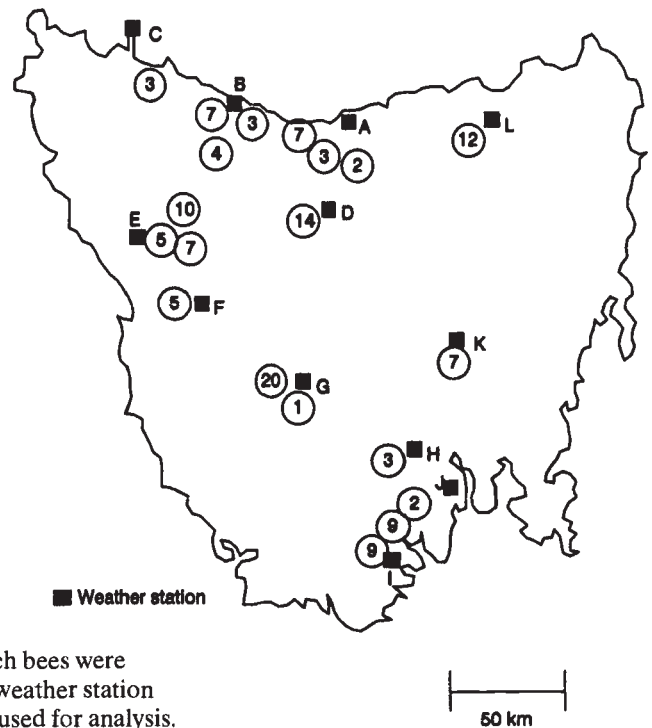
Fig. 1 Sites from which bees were collected, the nearest weather station and the seven regions used for analysis.

■ Weather station

50 km

Numbers in the circles indicate the number of colonies sampled at that apiary or site. Elevation (m) and long-term average monthly maximum and minimum temperatures (°C) for each station are as follows:

	Elevation	Feb T_{min}	Feb T_{max}	July T_{min}	July T_{max}	Region
A. Devonport	12	12.4	21.6	4.1	12.4	North coast
B. Burnie	10	12.9	21.0	5.5	12.5	North coast
C. Stanley	10	12.9	20.6	6.5	12.0	North coast
D. Deloraine*	253	10.3	22.3	1.5	10.5	(Commercial)
E. Rosebery	140	9.1	21.8	2.3	10.9	Tullah
F. Queenstown	129	8.4	22.0	2.3	11.6	Queenstown
G. Tarraleah	589	6.3	19.1	0.0	8.2	Tarraleah
H. New Norfolk	5	11.3	24.0	1.9	10.9	South coast
I. Geeveston	40	8.7	21.9	1.3	11.7	South coast
J. Kingston	52	10.7	21.9	2.4	12.5	South coast
K. Oatlands	400	8.6	21.9	1.1	9.4	Midlands
L. Launceston	170	10.2	23.0	2.3	10.8	Launceston

*Commercial apiary not used in most analyses.

numerous in the area (L. Slater, personal communication). We therefore included bees from Mr Slater's apiary in the feral population for the analyses below.

A further 14 colonies were sampled from a commercial apiary as an example of typical commercial stock. Samples from the commercial apiary were of daughters of different queens.

Mitochondrial DNA RFLPs

Total nucleic acid was extracted from the thorax of one bee per colony in an adaptation of a cell lysis method outlined in Sambrook *et al.* (1989). Each thorax was ground in 350 μL of GIT (4.0 M guanidinium thiocyanate, 0.1 M Tris-HCl (pH 7.5), 1 per cent *b-*

mercaptoethanol) buffer until the solutions turned pale yellow. Homogenates were then left to lyse for 15 min on ice; 125 μL of ice-cold 7.5 M ammonium acetate was then added, the tubes were inverted 10 times and placed on ice for 5 min. Particulate matter was then precipitated by centrifugation (1400 *g* for 10 min at 4°C). Supernatants were pipetted into new tubes. To these new tubes, 200 μL of phenol (pH 7.5) was added and the tubes gently inverted 50 times. Tubes were then centrifuged at 15 000 *g* for 15 min to precipitate a viscous pellet. Pellets were removed using wide-bore pipette tips. The remaining solutions were extracted twice, first with 200 μL chloroform/isoamyl alcohol (24:1), followed by pure chloroform. DNA was then precipitated in two volumes (about 600 μL) of ice-cold absolute ethanol, followed by resting at -70°C for 15 min and centrifugation for 10 min at 15 000 *g* (4°C). Supernatants were then discarded and the pellets rinsed in 70 per cent ethanol and resuspended in 1 × TE buffer.

Aliquots (15 μL) of total DNA preparations were digested with *Eco*RI according to the manufacturer's directions (Boehringer Mannheim, Sydney). These enzymes cleave honey bee mitochondrial DNA at specific sequences which produce patterns of restricted DNA considered to distinguish *A. m. mellifera* from *A. m. ligustica/carnica* type bees (Smith & Brown, 1990; Sheppard *et al.*, 1991). Digested DNA was electrophoresed in 1 per cent agarose gels at 60 V for 3 h and visualized by staining with ethidium bromide (Oldroyd *et al.*, 1992).

Protein electrophoresis

Thoraces of 12 bees per colony were each ground in 100 μL of water. These extracts were electrophoresed for 20 min on cellulose-acetate gels (Helena Laboratories, Beaumont, TX) at 200 V in a 0.08 M Tris-EDTA-maleic acid running buffer (pH 8.2). Malate dehydrogenase isozymes (Sylvester, 1976) were visualized by coating gels with 6 mL 1 per cent molten agar to which 0.6 mL 0.1 M Tris-HCl (pH 8.0), 0.4 mL each of 0.04 M nicotinamide adenine dinucleotide and 1 M disodium malate (pH 7.0), and 0.2 mL each of 0.145 M methyl-thiazole blue and 0.065 M phenazine methosulphate had been added (Cornuet, 1979). Three alleles were scored as *fast, medium* and *slow* according to the amount of anodal migration. These alleles correspond to Mdh^{65}, Mdh^{80} and Mdh^{100}, respectively, of Sheppard & Berlocher (1984). Where genotypes were ambiguous, they were rerun against known standards on the same gel. Although there are additional *Mdh* alleles in some populations (e.g. Badino *et al.*, 1983; Sheppard & Berlocher, 1984, 1985), they were not observed in this study.

Morphology

The standard character set for the USDA-ID (Rinderer *et al.*, 1993) morphometric determination of honey bee race was measured on 10 bees from each colony. Reference data sets from *A. m. mellifera* and *A. m. ligustica* were obtained from N. Koeniger (Institut für Bienenkunde, Oberursel, Germany). From the 18 characters common to both data sets, a factorial discriminant analysis was performed (Oldroyd *et al.*, 1992), which produced discriminant functions that were then used to classify the bees of the present collection as being more similar to the *A. m. mellifera* or *A. m. ligustica* reference specimens. A second discriminant analysis was performed on all 23 characters of the bees of Tasmania alone, to detect population subdivision at the morphological level.
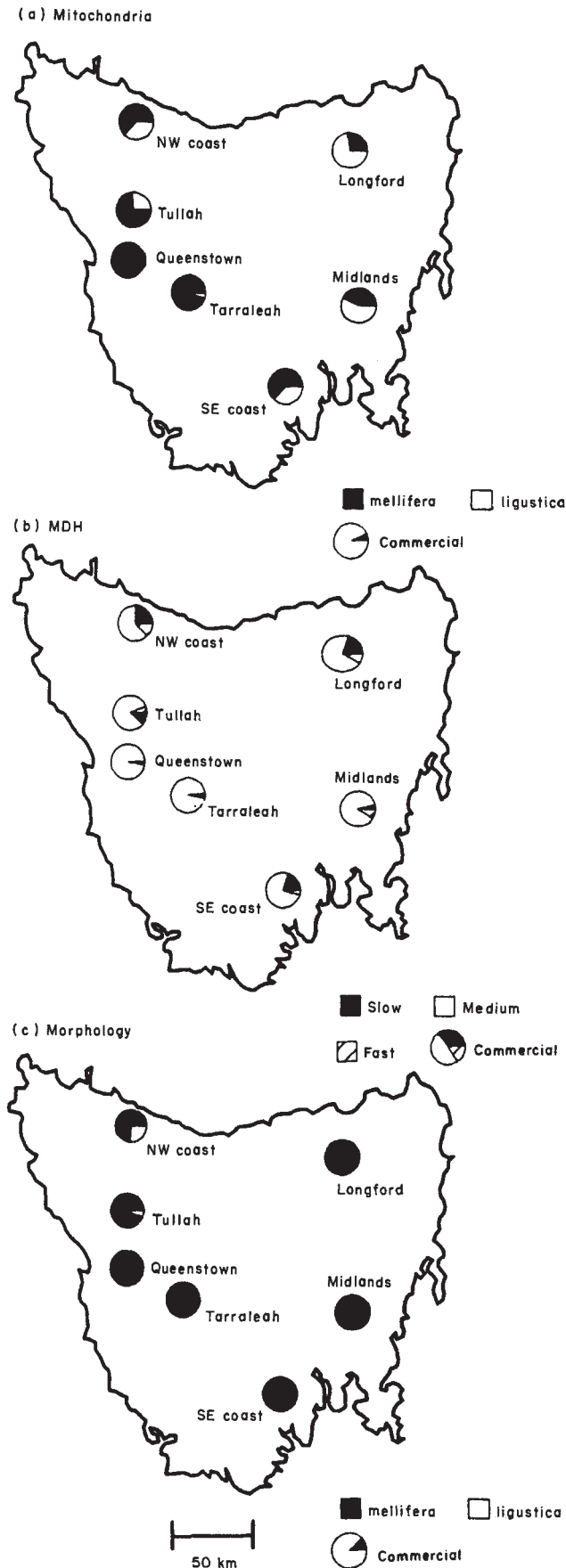
Environmental temperatures

Mean temperature minima (T_{min}) and maxima (T_{max}) for the coldest (July) and warmest (February) months were obtained from the Australian Bureau of Meteorology for the nearest weather station to each collection site (Fig. 1).

Results

Mitochondrial DNA

After digestion with *Eco*RI, two different haplotypes were found, one characteristic of *A. m. ligustica/ carnica* and the other of *A. m. mellifera* (Smith & Brown, 1990; Sheppard *et al.*, 1991). The feral populations sampled were divided into seven groups according to geographical location and elevation (Fig. 1). The relative frequencies of the two *Eco*RI haplotypes for these seven groupings are given in Fig. 2. The *mellifera* haplotype was at highest frequency in populations at higher elevation at Queenstown and Tarraleah. The *carnica/ligustica* haplotype was at highest frequency in the commercial population whereas bees that were collected in coastal areas had an intermediate frequency of the two types. The proportion of haplotypes was significantly heterogeneous among the seven feral populations ($\chi^2_6 = 19.5$, $P = 0.003$).

The frequency of the *mellifera* haplotypes in the population of colonies closest to each of the weather stations in Fig. 1 was computed and normalized with an arcsine $\sqrt{X}$ transformation as raw proportions data are not normally distributed (Steel & Torrie, 1980). There was no significant correlation between haplotype frequency and elevation or temperature (Table 1), probably because of the high frequency of the *carnica/ ligustica* mitotype at Tullah.

(a) Mitochondria

NW coast
Longford
Tullah
Queenstown
Midlands
Tarraleah
SE coast

■ mellifera   □ ligustica
◔ Commercial

(b) MDH

NW coast
Longford
Tullah
Queenstown
Midlands
Tarraleah
SE coast

■ Slow   □ Medium
▨ Fast   ◔ Commercial

(c) Morphology

NW coast
Longford
Tullah
Queenstown
Midlands
Tarraleah
SE coast

■ mellifera   □ ligustica
◔ Commercial

50 km

Mdh *allele frequencies*

All *Mdh* allele frequency data were normalized using an arcsine $\sqrt{X}$ transformation before analysis of correlation or variance. The frequencies of the three *Mdh* alleles were heterogenous (*slow* allele $P = 0.0001$, *medium* allele $P = 0.0001$, *fast* allele $P = 0.1$, ANOVA of the seven feral populations) over the island (Fig. 2). This heterogeneity was significantly associated with temperature (Table 1). The frequency of the *medium* allele was negatively correlated with temperature, the frequency of the *slow* allele positively correlated with temperature and the frequency of the *fast* allele was uncorrelated with temperature (Table 1). *Slow* and *medium* allele frequencies were also significantly correlated with elevation, which was significantly correlated with T_{min} and T_{max} in all months ($P < 0.05$).

When multiple tests of individual significance of correlation are performed, there is a danger of incorrectly declaring significance because of type I error. Therefore a sequential Bonferroni test (Rice, 1989) was performed on the correlations of Table 1. This test adjusts the probability that would be declared significant according to the number of simultaneous comparisons made. Because of the large number of correlations and low power of the test, this more conservative analysis did not reveal any significant correlations with an overall significance level of 5 per cent. Therefore, in a separate analysis, all environmental variables were combined into the first factor from a principal component analysis. By reducing the number of correlations examined simultaneously to five (Table 1) the type I error rate is reduced. The sequential Bonferroni test was performed on these five correlations and revealed that the *slow* allele frequency and the average discriminant score were significantly ($P < 0.05$) correlated with factor 1. The *medium* allele frequency was only marginally significant ($P < 0.1$).

Mdh genotype frequencies were tested for departures from Hardy–Weinberg equilibrium for each of the seven feral and one commercial populations using the algorithm of Guo & Thompson (1992) for Fisher's exact test. For this analysis, one bee was randomly selected from each colony. This avoids the bias created

Fig. 2 Distributions of mitochondrial haplotypes, *Mdh* genotypes and morphological phenotypes in the *Apis mellifera* population of Tasmania. (a) Proportions of queens carrying the *mellifera* and *ligustica* mitotypes as determined by restriction of mitochondrial DNA by *Eco*RI. (b) Relative frequencies of three alleles of *Mdh*-1. (c) Proportions of colonies which are more similar to *A. m. ligustica* and *A. m. mellifera* reference specimens according to the discriminant functions of Oldroyd *et al.* (1992).

Table 1 Correlation coefficients (with P in brackets) for mean temperatures, elevation at the nearest weather station ($n = 11$) and factor 1 from a principal component factor analysis of all these environmental variables with *Mdh* allele frequency, mtDNA RFLP frequency and discriminant score (function of Oldroyd *et al.*, 1992) for 114 honey bee colonies in Tasmania

Genetic marker or measure	Factor 1	Elevation	Feb T_{min}	Feb T_{max}	July T_{min}	July T_{max}
Frequency of *mellifera* mitochondria	−0.058 (0.86)	0.041 (0.90)	−0.17 (0.61)	−0.41 (0.21)	0.18 (0.60)	0.021 (0.95)
Frequency of colonies classified as *mellifera*-like by discriminant analysis	0.73* (0.01)	0.63 (0.04)	−0.81 (0.003)	0.01 (0.97)	−0.69 (0.01)	−0.67 (0.02)
Frequency of *slow* *Mdh* allele	0.77* (0.005)	−0.79 (0.004)	0.77 (0.005)	0.28 (0.39)	0.52 (0.10)	0.71 (0.01)
Frequency of *medium* *Mdh* allele	−0.66 (0.03)	0.61 (0.04)	−0.75 (0.007)	−0.19 (0.56)	−0.48 (0.13)	−0.57 (0.07)
Frequency of *fast* *Mdh* allele	0.30 (0.38)	−0.12 (0.72)	0.54 (0.08)	−0.0008 (1.0)	0.35 (0.29)	0.13 (0.69)

*Significant correlation ($P < 0.05$) as determined by a sequential Bonferroni test (Rice, 1989) of correlations between factor 1 and the 5 genetic parameters.

by queen genotype in cases where several workers are sampled per hive. There were no significant departures from Hardy–Weinberg equilibrium for any subpopulation ($P > 0.5$) or overall ($P > 0.99$).

Morphology

Colonies were classified according to their disciminant score as being more similar to *A. m. mellifera* or *A. m. ligustica* reference collections using the discriminant function described in Oldroyd *et al.* (1992). Nearly all bees were more similar to *A. m. mellifera* reference specimens than to *A. m. ligustica* reference specimens. Only commercial bees and a few collections from the north coast area were more similar to *ligustica* reference specimens than to *mellifera* ones (Fig. 2).

No Tarraleah bees were classified as being more similar to the *ligustica* reference specimens, all being classified as being of the *mellifera* type (Fig. 3). These results suggest that the *mellifera*-type morphology is conserved in the Tarraleah bees. The commercial bees sampled appear to be predominantly *ligustica*, but with some overlap with the *mellifera* type.

A cross validation study was conducted to examine population subdivision on the eight (seven feral and one commercial) populations in Tasmania at the morphometric level. Each colony was held out of the analysis while a new discriminant analysis was computed, and the held-out colony was classified by the new function. These analyses showed that the feral populations could not be reliably separated by discriminant analysis, with an overall error rate of 42 per cent. Classification error rates for each population ranged from 19 to 83 per cent, suggesting homogeneity of the feral population in terms of morphology. The

feral population is therefore quite homogeneous, although the Tarraleah population showed greatest separation from the commercial population on the basis of the Mahalanobis distance. Only the commercial bees showed reasonable separation from the other populations, and even here there was considerable overlap with some of the feral populations, with 13.3 per cent of commercial colony classifications being incorrect.

There were no significant individual correlations between any morphometric measurement and maximum temperatures in February (Table 2). No measures of wing size were significantly correlated with temperature or elevation. However, measures of appendage length (the leg measures; Table 2) showed significant negative individual correlation with February T_{min}, July T_{max} and July T_{min}, namely legs were significant negative individual correlations with February T_{min}, July T_{max} and July T_{min}, namely legs were 2) showed a number of significant negative correlations with temperature, suggesting larger bees in cooler areas. When all individual correlations were considered with the sequential Bonferroni test, there were no significant correlations at the 5 per cent level (Table 2). Even when the environmental variables were combined into a single factor score, wax mirror width B was the only individual morphometric measure to be significantly correlated with the combined environmental variables.

Associations among genetic markers

Within the feral population (110 colonies analysed) there was no significant association between bees classified as being more *mellifera* or *ligustica*-like on
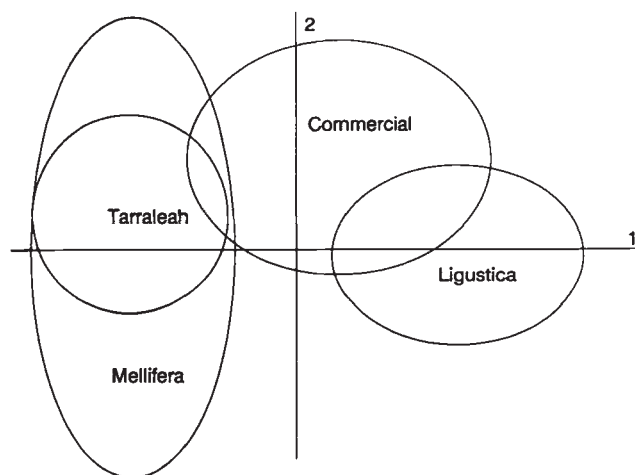
Fig. 3 Discriminant function analysis of the Tarraleah and commercial populations of *Apis mellifera*. Discriminant functions were produced from a discriminant analysis of *A. m. ligustica* and *A. m. mellifera* reference specimens from Europe (Oldroyd *et al.*, 1992) and 95 per cent confidence ellipses drawn by the method of Cornuet (1982). The Tarraleah and commercial populations were then classified by this analysis. The Tarraleah population lies within the *mellifera* reference specimens whereas the commercial bees have evidence of hybridization between the two types.

the basis of morphology and their mitochondrial haplotype ($P > 0.25$, d.f. = 1, *G*-test of homogeneity). However, there were strong associations between discriminant score and colony frequency of the three *Mdh* alleles (*fast* allele, $r = -0.41$, $P < 0.001$; *medium*, $r = 0.60$, $P < 0.001$; *slow*, $r = -0.53$, $P < 0.0001$).

A nonrandom association of mtDNA and allozyme genotypes exists across the island (Table 3). Asmussen *et al.* (1987) provide statistics to partition such disequilibrium between nuclear and mtDNA markers to make inferences about the process of hybridization between two subspecies. They define an allelic disequilibrium (D) which measures overall associations between the nuclear and mtDNA genotypes, and three genotypic disequilibria (D_1, D_2, D_3) which correspond to associations between two mtDNA genotypes, and three nuclear genotypes. We computed these D statistics after pooling the *fast* and *slow* alleles (Table 4). Again, there were highly significant associations between the nuclear and mtDNA markers ($P = 0.0042$, Fisher's exact test). Examination of the D values reveals that in homozygous *medium* individuals, there was a higher than expected frequency of the *mellifera* mitotype whereas in heterozygous *medium* individuals, there was a lower than expected frequency of the *mellifera* mitotype.

Discussion

Of the parameters measured, the best overall measure of racial type is morphology because of the variety and extremely high heritability of the characters used (Oldroyd *et al.*, 1991). According to this criterion, the feral bees of Tasmanian appear strongly *mellifera*-like in the cooler regions at Queenstown and Tarraleah, and more *lugustica*-like in warmer north-coastal regions (Fig. 2). The commercial bees sampled were more *ligustica*-like than *mellifera*-like in their morphology (Fig. 2) and all other measures (Figs 2 and 3). The distribution of mitochondrial DNA haplotypes and *Mdh* allele frequencies suggest that hybridization between *A. m. mellifera* and *A. m. ligustica* is extensive, particularly in coastal areas. The mating biology of the species and the fact that all subpopulations were in Hardy–Weinberg proportions suggest that all populations were random mating. At Queenstown and Tarraleah, there is only a slight influence of *ligustica* genotypes as revealed by low mt-DNA and *Mdh* markers normally associated with *A. m. ligustica*, and the significant D values (Table 4).

This heterogeneity in the population is not caused by physical separation of coastal and highland populations. The major honey-producing areas of Tasmania are in the western highlands (the Queenstown and Tullah sample sites are within this area), and tens of thousands of commercial bee colonies are moved into the area for summer production. Thus although the commercial colonies are moved in after the swarming season, large numbers of *ligustica*-type drones are present in the area every year, providing the opportunity for hybridization. Given that Africanized bees were able to spread through south and central America at the rate of 200–400 km per year (Taylor, 1977), the 100 years of active propagation of *ligustica*-type bees by apiarists would seem enough time for *ligustica* alleles to become widely spread across the 300 km long island unless there are genetic impediments to that spread.

We conclude that *A. m. mellifera* and *A. m. ligustica* form hybrids which have fitness equal to or greater than that of the parents in coastal environments. Gene flow occurs between the commercial, predominantly *ligustica* population into the feral, predominantly *mellifera* population on the coast, but is restricted in cooler regions where there is a very large feral population of *A. m. mellifera*. We speculate that the fitness of *A. m. ligustica* and *ligustica/mellifera* hybrids is lower than that of 'pure' *A. m. mellifera* in cooler regions. There is some exchange of selectively neutral mitochondria between the populations, but less movement of the *Mdh* alleles which may not be selectively neutral in different climates (Nielson *et al.*, 1994; Cornuet *et*

Table 2 Correlation coefficients (with P in brackets) for mean temperatures, elevation at the nearest weather station ($n = 11$) and factor 1 from a principal component factor analysis of all these environmental variables with various morphometric measures defined by Rinderer *et al.* (1993)

	Factor 1	Elevation	Feb T_{min}	Feb T_{max}	July T_{min}	July T_{max}
Forewing length	−0.400 (0.22)	0.494 (0.12)	−0.317 (0.34)	−0.213 (0.53)	−0.076 (0.82)	−0.511 (0.11)
Forewing width	−0.480 (0.13)	0.551 (0.08)	−0.480 (0.13)	−0.433 (0.18)	−0.113 (0.74)	−0.462 (0.15)
Hindwing length	−0.028 (0.93)	0.113 (0.74)	−0.057 (0.87)	−0.328 (0.32)	0.357 (0.28)	−0.105 (0.76)
Hindwing width	0.165 (0.63)	−0.077 (0.82)	0.193 (0.57)	−0.217 (0.52)	0.374 (0.26)	0.111 (0.74)
Cubital vein B	−0.698 (0.02)	0.648 (0.03)	−0.741 (0.009)	−0.123 (0.72)	−0.575 (0.06)	−0.646 (0.03)
Cubital vein A	0.657 (0.03)	−0.506 (0.11)	0.784 (0.004)	−0.020 (0.95)	0.748 (0.008)	0.508 (0.11)
Tibia length	−0.713 (0.01)	0.666 (0.02)	−0.761 (0.006)	0.035 (0.92)	−0.661 (0.03)	−0.659 (0.03)
Femur length	−0.568 (0.07)	0.507 (0.11)	−0.594 (0.05)	0.163 (0.63)	−0.558 (0.07)	−0.593 (0.05)
Basitarsis length	−0.123 (0.72)	0.200 (0.55)	−0.101 (0.77)	0.144 (0.67)	−0.116 (0.73)	−0.118 (0.73)
Basitarsis width	−0.020 (0.95)	0.128 (0.71)	−0.037 (0.91)	0.096 (0.78)	0.175 (0.61)	−0.107 (0.75)
Sternite length	−0.409 (0.21)	0.362 (0.27)	−0.387 (0.24)	0.098 (0.77)	−0.280 (0.40)	−0.570 (0.07)
Wax mirror length	−0.394 (0.23)	0.376 (0.25)	−0.416 (0.20)	−0.027 (0.93)	−0.223 (0.51)	−0.466 (0.15)
Wax mirror width A	−0.681 (0.02)	0.658 (0.03)	−0.620 (0.04)	0.158 (0.64)	−0.667 (0.02)	−0.734 (0.01)
Wax mirror width B	0.775* (0.005)	−0.638 (0.034)	0.879 (0.0004)	0.119 (0.73)	0.796 (0.003)	0.614 (0.04)

*Significant ($P < 0.05$) correlation as determined by a sequential Bonferroni test (Rice, 1989) of correlations between factor 1 and the 12 morphometric measures.

Table 3 Association of mtDNA genotypes with allozyme genotypes for 109 feral colonies of honey bees in Tasmania†

	Observed (and expected) numbers of mt-DNA genotypes‡	
Mdh genotype	*mellifera*	*ligustica*
Slow/slow	0(0.68)	1(0.31)
Slow/medium	10(13.76)	10(6.23)
Slow/fast	1(2.75)	3(1.24)
Medium/medium	58(51.6)	17(23.39)
Medium/fast	5(6.19)	4(2.81)
Fast/fast§	—	—

†The probability of observing a table with at least this much association (Fisher's exact test) is 0.018.
‡Expected frequencies computed from observed population-wide frequencies.
§*Fast/fast* genotype absent from this sample.

al., 1994). This, therefore, is an example of secondary contact where fitness is affected by an environmental gradient.

What do our Tasmanian results reveal about the honey bee populations of Europe, the original home of *A. m. mellifera* and *A. m. ligustica*, and the hybrid zones between these subspecies? Clearly, the bees of Tasmania have established a similar population structure to their ancestors in Europe. One is tempted to suggest that the population has subdivided in Tasmania in parallel with similar environments in Europe. This scenario fits well with the situation in the mountains of northern Italy, but does not explain the hybrid zone between *ligustica* and *mellifera* on the temperate Ligurian coast where there is little or no temperature gradient across the hybrid zone, at least along the coast.

Hybrid zones which are the result of recent contact should broaden over time as there is an exchange of

Table 4 Genotypic frequencies (expected†) and cytonuclear disequilibria between *Mdh* and mtDNA genotypic frequencies for 109 feral honeybee colonies in Tasmania

	Mdh genotypic frequencies§		
	AA	*AB*	*BB*
mtDNA			
mellifera	2(2.75)	15(21.33)	58(50.9)
ligustica	2(1.25)	16(9.67)	16(23.08)
Disequilibria‡ ± SE			
	D_1	D_2	D_3	D
	-0.069 ± 0.096	$-0.058 \pm 0.021^*$	$0.065 \pm 0.022^*$	$0.035 \pm 0.012^*$

†Expected frequencies computed from population frequencies of mtDNA types.
‡D values computed according to Asmussen *et al.* (1987).
§A, *slow+fast*; B, *medium*.
*Significantly different from 0 ($P < 0.05$).

genes between the two populations (Barton & Hewitt, 1985). In the absence of fitness differences, the width of such zones should increase proportionally to the number of generations, G, since contact and the variance of the dispersal distance of the subject organism per generation, σ^2, such that W, the width of the cline, is given by:

$$W = \sqrt{(G2\pi/\sigma)} \tag{1}$$

(rearranged from Barton & Hewitt, 1985).

If we assume contact between *A. m. mellifera* and *A. m. ligustica* approximately 10 000 years ago (after the last glaciation), then G = about 10 000. If honey bee swarms disperse at 0–2 km per generation (Lindauer, 1961), then σ is probably about 1. According to eqn 1 an approximately 250 km wide hybrid zone should exist between the two subspecies in Europe. This fits fairly well with the observed width (Cornuet, 1983; Badino *et al.*, 1983, 1984, 1988a,b; Comparini & Biasiolo, 1991; Nazi, 1992; Meixner *et al.*, 1993), but cannot explain the apparent racial admixture in Tasmania, where there is artificial mixing of genotypes by beekeepers.

Alternative hypotheses to explain hybrid zones relate to hybrid dysfunction or superiority in the narrow environment of the tension zone. Harrison & Hall (1993) suggested that incompatibilities between mitochondria and cytoplasm in the European and African bees might reduce hybridization between European and Africanized populations. A mechanism such as this is required for the formation of a 'tension zone' (Barton & Hewitt, 1985). Our data show that such a mechanism is not operating in Tasmania. Even at the coldest, most isolated site (Tarraleah) the *ligustica* mitotype was identified in a colony that was strongly

mellifera-like in terms of morphology and *Mdh* allele frequency. Such colonies can only exist after repeated generations of backcrossing and should be eliminated if a significant incompatibility exists between *ligustica* mitochondria and the *mellifera* nuclear genome (Rinderer *et al.*, 1991).

Inter-racial crosses between honey bees generally result in increased fitness in F_1s (Cale & Gowen, 1956; Ruttner, 1968; Oldroyd *et al.*, 1985). Therefore hybrid depression in a tension zone is unlikely to impede gene flow between these subspecies. Thus we can eliminate both popular explanations of hybrid zones (recent contact and hybrid dysfunction; Hewitt, 1988) for the apparent clines between *A. m. mellifera* and *A. m. ligustica* in Tasmania.

Restriction of gene flow between populations may also result from assortative mating. Koeniger *et al.* (1989) found assortative mating between *A. m. ligustica* and *A. m. carnica* in the Austrian Alps. We hypothesize that *mellifera* drones and queens will fly in cooler weather than *ligustica*-type drones and queens, at least in Tasmania. This would reduce inter-racial mating in cooler areas, but allow hybridization in the coastal areas. If this hypothesis is correct, then the maintenance of the zones of hybridization and the lack of gene flow between *A. m. carnica, A. m. ligustica* and *A. m. mellifera* in the European Alps may be explained by this same phenomenon.

Nielson *et al.* (1994) demonstrated that on three continents, the frequency of the *Mdh fast* allele is positively correlated with average environmental temperature, the frequency of the *medium* allele negatively correlated with temperature and the frequency of the *slow* allele only weakly correlated with temperature. In contrast, we found only a weak negative corre-

lation between temperature and *medium* allele frequency, but a significant correlation with frequency of the *slow* allele. Nielson *et al.* (1994) argued that the existence of parallel clines for *Mdh* relative allele frequency associated with environmental temperature in Spain, Brazil and California is strong evidence for direct selection on the allelic forms. Cornuet *et al.* (1994) demonstrated differing *in vitro* thermostabilities of the allelic forms thus suggesting a mechanism by which selection could act to develop such clines. Our results also show that *Mdh* allele frequency and discriminant scores are correlated with environmental temperature in Tasmania (Tables 1 and 2). However, this is not evidence for direct selection on allelic frequency or the lengths and sizes of various appendages. Rather, our correlations seem to result from an abrupt change from the *mellifera*-type on the highland sites to a hybridized *ligustica*-type on the (particularly northern) coast. This explanation accounts for the correlation of discriminant score with environmental variables that must otherwise be explained by selection, which seems unlikely (Rinderer *et al.*, 1991). But, the lack of a selectively based cline for the *medium* allele in Tasmania does not preclude the existence of such clines within racial groups elsewhere. However, at least in Brazil, Italy and Spain, the clines may be equally explained by competitive displacement of racial types.

Finally, our morphological and molecular data support the findings of Ruttner (1976) which suggest that the Tarraleah population is a well preserved population of *A. mellifera mellifera*, having only a weak genetic component derived from other sources.

Acknowledgements

References

ARNOLD, J. 1993. Cytonuclear disequilibria in hybrid zones. *Ann. Rev. Ecol. Syst.*, **24**, 521–554.

ASSMUSSEN, M. A., ARNOLD, J. AND AVISE, J. C. 1987. Definition and properties of disequilibrium statistics for associations between nuclear and cytoplasmic genotypes. *Genetics*, **115**, 775–768.

AYTON, H. 1977. The black bees of Tarraleah. In: Harnaj, V., Doull, K. and Harnaj, A. (eds) *XXVI International Congress of Apiculture*, pp. 266–268. Apimondia Publishing House, Bucharest.

BADINO, G., CELEBRANO, G. AND MANINO, A. 1983. Population structure and *MDH-1* locus variation in *Apis mellifera ligustica*. *J. Hered.*, **74**, 443–446.

BADINO, G., CELEBRANO, G. AND MANINO, A. 1984. Population genetics of Italian honeybee (*Apis mellifera ligustica* Spin.) and its relationships with neighbouring subspecies. *Bol. Mus. Reg. Sci. Nat. Torino*, **2**, 571–584.

BADINO, G., CELEBRANO, G., MANINO, A. AND IFANTIDUS, M. D. 1988a. Allozyme variability in Greek honey bees (*Apis mellifera* L.) *Apidologie*, **19**, 377–386.

BADINO, G., CELEBRANO, G. AND MANINO, A. 1988b. Genetica di popolazione delle sottospecie mediterranee di *Apis mellifera* L. sulla base di varianti alloenzimatiche. *Apicult. Mod.*, **79**, 233–239.

BARTON, N. H. AND HEWITT, G. M. 1985. Analysis of hybrid zones. *Ann. Rev. Ecol. Syst.*, **16**, 113–148.

BAZYKIN, A. D. 1969. A hypothetical mechanism of speciation. *Evolution*, **23**, 685–687.

CALE, G. H. AND GOWEN, J. W. 1956. Heterosis in the honey bee (*Apis mellifera* L.) *Genetics*, **41**, 292–303.

COMPARINI, A. AND BIASIOLO, A. 1991. Genetic discrimination of Italian bee *Apis mellifera ligustica* from Carniolan bee, *Apis mellifera carnica* by allozyme variability analysis. *Bio. Syst. Ecol.*, **19**, 189–194.

CORNUET, J.-M. 1979. The MDH system of the honey bees of Guadaloupe. *J. Hered.*, **70**, 223–224.

CORNUET, J.-M. 1982. Représentation graphique de populations multinormales par des ellipses de confiance. *Apidologie*, **13**, 15–20.

CORNUET, J.-M. 1983. Reproduction génétique et sélection de l'abeille. *Bull. Tech. Apic.*, **10**, 13–36.

CORNUET, J.-M., OLDROYD, B. P. AND CROZIER, R. H. 1994. Unequal thermostability of allelic forms of malate dehydrogenase in honey bees. *J. Apic. Res.* (in press).

DALY, H. V., HOELMER, K. AND GAMBINO, P. 1991. Clinal geographic variation in feral honey bees in California, USA. *Apidologie*, **22**, 591–607.

ENDLER, J. A. 1977. *Geographic Variation, Speciation and Clines.* Princeton University Press, Princeton, New Jersey.

GARNERY, L. 1992. *Variabilité de l'ADN mitochondrial de l'abeille domestique implications phylogénétiques.* Ph.D. Thesis, Université de Paris.

GARNERY, L., CORNUET, J.-M. AND SOLINAC, M. 1992. Evolutionary history of the honey bee *Apis mellifera* inferred from mitochondrial DNA analysis. *Mol. Ecol.*, **1**, 145–154.

GUO, S. W. AND THOMPSON, E. A. 1992. Performing an exact test of Hardy–Weinberg proportion for multiple alleles. *Biometrics*, **48**, 361–372.

HARRISON, J. F. AND HALL, H. G. 1993. African–European honeybee hybrids have low nonintermediate metabolic capacities. *Nature*, **363**, 258–260.

HEWITT, G. M. 1988. Hybrid zones: natural laboratories for evolutionary studies. *Trends Ecol. Evol.*, **3**, 158–167.

KOENIGER, G., KOENIGER, N., PECHHACKER, H., RUTTNER, F. AND BERG, S. 1989. Assortative mating in a mixed population of European honeybees, *Apis mellifera ligustica* and *Apis mellifera carnica*. *Ins. Soc.*, **36**, 129–138.

LINDAUER, M. 1961. *Communication Among Social Bees*. Harvard University Press, Cambridge, MA.

MEIXNER, M. D., SHEPPARD, W. S. AND POKLUKAR, J. 1993. Asymmetrical distribution of a mitochondrial DNA polymorphism between two introgressing honey bee species. *Apidologie*, **24**, 147–153.

MOORE, W. S. 1977. An evaluation of narrow hybrid zones in vertebrates. *Quart. Rev. Biol.*, **52**, 263–277.

MOORE, W. S. AND BUCHANAN, D. B. 1985. Stability of the northern flicker hybrid zone in historical times: implications for adaptive speciation theory. *Evolution*, **39**, 135–151.

NAZI, F. 1992. Morphometric analysis of honey bees from an area of racial hybridization in northeastern Italy. *Apidologie*, **23**, 89–96.

NIELSON, D., PAGE, R. E. AND CROSLAND, M. W. J. 1994. Clinal variation and selection of MDH allozymes in honey bee populations. *Experientia*, **50**, 867–871.

OLDROYD, B. P., MORAN, C. AND NICHOLAS, F. W. 1985. Diallel crosses of honey bees. 1. Genetic analysis of honey production using a fixed effects model. *J. Apic. Res.*, **24**, 243–249.

OLDROYD, B. P., RINDERER, T. E. AND BUCO, S. M. 1991. Heritability of morphological characters used to distinguish European and Africanized honeybees. *Theor. Appl. Genet.*, **82**, 499–504.

OLDROYD, B. P., SHEPPARD, W. S. AND STELZER, J. A. 1992. Genetic characterisation of the bees of Kangaroo Island, South Australia. *J. Apic. Res.*, **31**, 141–148.

RICE, W. R. 1989. Analysing tables of statistical tests. *Evolution*, **43**, 223–225.

RINDERER, T. E., STELZER, J. A., OLDROYD, B. P., BUCO, S. M. AND RUBINK, W. L. 1991. Hybridization between European and Africanized honey bees in the neotropical Yucatan Peninsula. *Science*, **253**, 309–311.

RINDERER, T. E., BUCO, S. M., RUBINK, W. L., DALY, H. V., STELZER, J. A., RIGGIO, R. M. AND BAPTISTA, F. C. 1993. Morphometric identification of Africanized and European honey bees using large reference populations. *Apidologie*, **24**, 569–585.

RUTTNER, F. 1968. Methods of breeding honey bees: intraracial selection or inter-racial hybrids? *Bee World*, **49**, 66–72.

RUTTNER, F. 1976. Isolated populations of honeybees in Australia. *J. Apic. Res.*, **15**, 97–104.

RUTTNER, F. 1988. *Biogeography and Taxonomy of Honeybees*. Springer, Berlin.

RUTTNER, F., TASSENCOURT, L. AND LOUVEAUX, J. 1978. Biometrical-statistical analysis of the geographical variability of *Apis mellifera* L. *Apidologie*, **9**, 363–381.

SAMBROOK, J., FRITSCH, E. F. AND MANIATIS, T. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

SHEPPARD, W. S., RINDERER, T. E., MAZZOLI, J. A., STELZER, J. A. AND SHIMANUKI, H. 1991. Gene flow between African- and European-derived honey bee populations in Argentina. *Nature*, **349**, 782–784.

SHEPPARD, W. S., AND BERLOCHER, S. H. 1984. Enzyme polymorphism in *Apis mellifera* from Norway. *J. Apic. Res.*, **23**, 64–69.

SHEPPARD, W. S. AND BERLOCHER, S. H. 1985. New allozyme variability in Italian honey bees. *J. Hered.*, **76**, 45–85.

SMITH, D. R. 1991. African bees in the Americas: insights from biogeography and genetics. *Trends Ecol. Evol.*, **6**, 17–21.

SMITH, D. R. AND BROWN, W. M. 1990. Restriction endonuclease cleavage site and length polymorphisms in mitochondrial DNA of *Apis mellifera mellifera* and *A. m. carnica* (Hymenoptera: Apidae). *Ann. Ent. Soc. Am.*, **83**, 81–88.

STEEL, R. D. G. AND TORRIE, J. H. 1980. *Principles and Procedures of Statistics*, 2nd edn. McGraw-Hill Kogakusha, Tokyo.

SYLVESTER, H. A. S. 1976. Allozyme Variation in Honeybees (*Apis mellifera*). Ph.D. Thesis, University of California at Davis.

TAYLOR, O. R. 1977. The past and possible future spread of Africanized honeybees in the Americas. *Bee World*, **58**, 38–47