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**Original article**

# Genetic characterization of Italian honeybees, *Apis mellifera ligustica*, based on microsatellite DNA polymorphisms*

Raffaele DALL'OLIO[a], Alberto MARINO[a], Marco LODESANI[a], Robin F.A MORITZ[b]

[a] Istituto Nazionale di Apicoltura, via di Saliceto, 80 40128 Bologna, Italia
[b] Institut für Zoologie, Martin-Luther-Universität Halle-Wittenberg, Hoher Weg 4, 06099 Halle/Saale, Germany

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**Abstract** – The genetic variability of *Apis mellifera ligustica* was screened throughout the Italian peninsula and Sardinia with eight polymorphic microsatellite loci. Samples of *Apis mellifera mellifera*, *Apis mellifera carnica* and from the Buckfast breeding line were genotyped for comparison. Low *Fis* and *Fst* values suggest the absence of genetic structure within and among *A. m. ligustica* populations, although the high number of alleles detected and heterozygosity. Phylogenetic and individual analyses confirmed that *A. m. ligustica* has come to resemble one large population, probably as a result of intensive beekeeping practices such as migratory beekeeping and large-scale commercial queen trading. Since the introgression of foreign alleles into both endemic natural and commercial *A. m. ligustica* populations, can be detected and monitored by microsatellite analysis, the results provide a reference data set for future local biodiversity conservation and other controlled breeding programs.

***Apis mellifera ligustica* / microsatellite / genetic variability / conservation**

## 1. INTRODUCTION

The honeybee *Apis mellifera* L. has been intensively studied to assess both its phylogenetic origin and taxonomy. Ruttner (1988) conducted the most extensive morphological characterization, classifying 24 *Apis mellifera* subspecies into four major evolutionary biogeographic branches. Ruttner's now classic hypothesis is supported by a set of morphometric studies (Cornuet and Fresnaye, 1989; Cornuet et al., 1988; Lebdigrissa et al., 1991). Three main evolutionary branches, 'A' (African subspecies), 'C' (northern Mediterranean subspecies, including the Italian honeybee, *Apis mellifera ligustica* Spinola) and 'M' (western European subspecies) have been defined using mitochondrial DNA (Garnery et al., 1992; Franck et al., 1998) and nuclear markers (Estoup et al., 1995). The mitochondrial C branch is composed of subspecies from both the "C" and "O" morphological lineages, suggesting that the differentiation of these lineages postdated the origin of the C mitochondrial branch.

The Italian peninsula plays a pivotal role for honeybee biogeography in this scenario, as it did for many other taxa during the Pleistocene glacial period (Hewitt, 1996; Taberlet et al., 1998; Hewitt, 1999). *A. m. ligustica* genetic variability has been studied in detail using allozymes (Badino et al., 1982, 1983; Manino and Marletto, 1984; Marletto et al., 1984) and other molecular markers (Franck, 2004), which have shown hybridization with *A. m. mellifera* along the western Alpine arc and the Ligurian coast. Moreover, the eastern Alpine arc and particularly the Friuli region were already known as a natural hybridization zone with a subspecies from the 'C' lineage, *Apis mellifera carnica* (Comparini and Biasiolo, 1991; Nazzi, 1992; Meixner

Corresponding author: R. Dall'Olio,
rdallolio@inapicoltura.org
* Manuscript editor: Walter S. Sheppard

et al., 1993). Recently, extensive studies have also provided mitochondrial and microsatellite evidence of past contacts between honeybees from different evolutionary branches, thus confirming past hybridization within Italian honeybees (Franck et al., 2000; Marino et al., 2002).

Italian beekeeping developed from an ancient rural tradition. Today the queen bee breeding system is based on selection within autochthonous populations. The endemic subspecies has provided many advantages for beekeeping practice due to its large honey storing ability, docility and low swarming propensity. Because of these traits and the subspecies' adaptability to a wide range of climatic conditions, *A. m. ligustica* queens have been massively exported worldwide for more than 150 years (Bar-Cohen et al., 1978; Sheppard, 1989; Woodward, 1993). In spite of this success, the importation of non-*ligustica* queens into Italy has also increased, particularly during the last ten years (source: Italian Health Ministry, B.I.P. – Border Inspection Post – data collection system). Besides reflecting the curiosity of beekeepers to work with new stocks, this increase is likely to be due to the lower production costs to be found elsewhere and the possibility of having queens right at the beginning of spring, if imported from the southern hemisphere. It is currently unclear whether or how these imports affect locally existing populations in Italy. Given the success and scale of the queen breeding industry, the conservation of Italian honeybees is of major concern even for economic reasons alone. However, its conservation is also important from a biodiversity perspective, where a priority is laid on preserving the endemic races of honeybees in Europe.

Here we present a nationwide data base which provides insight into the current genetic variability within *A. m. ligustica.* This data base will be an important tool for detecting introgression of genes from other subspecies and for determining the effects of a breeding policy based on controlled and co-ordinated rearing programs with the intense rearing of thousand of queens produced by selected mothers. We will also address the effects of professional honeybee breeding and the potential impacts of population bottlenecks caused by disease (Lodesani et al., 1995) on the population structures of *A. m. ligustica*.

**Table I.** Locus-specific PCR conditions; temperature ramping speed between cycle steps was 5 ˚C/s.

| Locus | [$MgCl_2$] | Cycles | Tm (˚C) |
|---|---|---|---|
| A113 | 1.0 mM | 30 | 60 |
| A7 | 1.2 mM | 25 | 58 |
| A88 | 1.2 mM | 30 | 55 |
| A(B)24 | 1.2 mM | 30 | 55 |
| A107 | 1.2 mM | 30 | 58 |
| Ap43 | 1.2 mM | 35 | 60 |
| A28 | 1.7 mM | 30 | 54 |
| A14 | 1.7 mM | 30 | 58 |

## 2. MATERIALS AND METHODS

### 2.1. Sampling

Samples were collected from beekeepers (mainly hobbyist beekeepers who exclusively used putative *A. m. ligustica* queens) or were kindly provided by researchers. A single worker per colony was trapped and immediately killed by immersion in absolute ethanol and then stored at –20 ˚C until laboratory processing. A total of 379 colonies were sampled in different locations over a four-year period (2001–2004). *A. m. ligustica* samples were initially assigned to seven distinct geographical groups (Fig. 1; exact sampling locations are available on the web, www.inapicoltura.org/online/apidologie2006/sample.htm). Three more groups were constituted with samples from *A. m. mellifera*, *A. m. carnica* and the Buckfast breeding line respectively. Taxa cited in the text in normal case letters (e.g. Carnica) specifically refer to a geographical group.

### 2.2. Molecular analyses

Total DNA was extracted from individual workers' anterior leg after rinsing twice with bi-distilled water. DNA isolation was performed in a 96-well microtitre plate, following the Chelex method (Walsh et al., 1991) with slight modifications. Eight polymorphic microsatellite loci were analysed: A113, A28, A(B)24, A14, A107, A88, Ap43 and A7 (Solignac et al., 2003). The PCR reaction mixture contained 1X *Taq* buffer (Invitrogen),


**Figure 1.** Geographic distribution of the 379 sampled colonies. Italian samples were pooled in seven regions. The non-*A. m. ligustica* samples were treated as three additional taxonomic groups; name and sample size for each of the 10 groups are indicated.

400 nM of each primer, 1 mM of each dNTP, 0.25 U of *Taq* DNA polymerase, 1 μL of DNA extract and PCR-water to a final volume 10 μL. $MgCl_2$ concentration and annealing temperature are reported in Table I, while PCR cycle conditions were standard (Garnery et al., 1998); Invitrogen oligo primer pairs were used and the forward primer was labelled with WellRED. PCRs were performed with a T-Gradient 96 thermocycler (Biometra) and PCR products were separated by capillary electrophoresis on a CEQ8000 Genetic Analysis System (Beckman Coulter, software ver. 8.0). Allele sizes were scored with the CEQ DNA Size Standard Kit-400 (Beckman Coulter).

### 2.3. Statistical analyses

Unbiased estimates and standard deviations of gene diversity of microsatellite loci were computed according to the method described by Nei (1978); genotypic linkage disequilibrium (with Bonferroni correction), population parameters and microsatellite variation within and between groups were analysed with "Fstat" software (Goudet, 1995). Exact tests for Hardy–Weinberg equilibrium and genotypic differentiation were carried out using the "Genepop" package (Raymond and Rouset, 1995 ver.3.1). Multiple test significance was assessed using Fisher's exact test (Genepop package). Genetic differentiation between groups was computed using unbiased estimates of *Fst* values provided by Fstat and the $(\delta\mu)^2$ microsatellite distance (Goldstein et al., 1995).

Neighbor-joining analyses were performed to establish relationships among groups using the "PHYLIP" software package (Felsenstein, 1993 ver.3.57), with the NJ-algorithm (Saitou and Nei, 1987) and the two genetic distances of Cavalli-Sforza and Edwards (1967) and Nei (1978). Bootstrap values were computed over 2000 replications (Hedges, 1992) by resampling either loci or individuals within populations.

"Structure" (Pritchard et al., 2000 ver.2.1) software was used to obtain individual 'a posteriori' confirmation of the taxonomic posting for each sample: a first analysis was conducted to infer the most likely number of population ($k$), representative of the whole dataset, without the use of any 'a priori' information; a sub-dataset including only *A. m. mellifera*, *A. m. carnica* and Emilia-Romagna (to represent *A. m. ligustica*) bees was subsequently investigated to detect the presence of hybrid traits in north-eastern Italian bees and assess the genetic composition of Buckfast samples. The 'Admixture Model' and 'Correlation Model' were the parameters chosen for the purposes of analysis; a 75% level of cut-off was used to validate the correct posting of each sample in the inferred populations.

**Table II.** Overall parameters of the 10 groups across all loci. N = mean sample size, n = mean observed number of alleles; *Ho* = observed heterozygosity; *He* = expected heterozygosity; the *Fis* values are also shown with the *P*-value after Bonferroni's correction (***: $P < 0.01$; *: $0.01 < P < 0.05$; n.s.: $P > 0.05$).

| Group name | N | n ± SE | *Ho* ± SE | *He* ± SE | *Fis* (*P*-value) |
|---|---|---|---|---|---|
| Emilia Romagna | 39 | 8 ± 4.717 | 0.495 ± 0.243 | 0.563 ± 0.255 | 0.136 * |
| Carnica | 48 | 6.6 ± 3.238 | 0.470 ± 0.228 | 0.528 ± 0.256 | 0.121 * |
| Mellifera | 11 | 5.3 ± 1.714 | 0.375 ± 0.213 | 0.599 ± 0.221 | 0.418 *** |
| Buckfast | 12.3 | 7 ± 3.391 | 0.605 ± 0.265 | 0.637 ± 0.236 | 0.093 n.s. |
| North east | 32.3 | 6.9 ± 3.179 | 0.534 ± 0.236 | 0.571 ± 0.216 | 0.080 n.s. |
| North west | 26.8 | 7.9 ± 3.370 | 0.556 ± 0.257 | 0.611 ± 0.237 | 0.111 * |
| Adriatic coast | 33.8 | 7.8 ± 3.527 | 0.478 ± 0.247 | 0.570 ± 0.276 | 0.161 *** |
| Mediterr. coast | 67.4 | 9.4 ± 4.897 | 0.504 ± 0.254 | 0.561 ± 0.251 | 0.110 *** |
| South | 27.6 | 7.3 ± 3.865 | 0.459 ± 0.234 | 0.526 ± 0.284 | 0.145 * |
| Sardinia | 17.9 | 5.5 ± 3.041 | 0.509 ± 0.277 | 0.542 ± 0.271 | 0.093 n.s. |

## 3. RESULTS

Overall parameters of the 10 groups and the *Fis* values are shown in Table II. Observed heterozygosities varied between 0.375 (*A. m. mellifera*) and 0.605 (Buckfast). The average number of alleles per locus per population ranged between n = 5.3 (Mellifera) and n = 9.4 (Mediterranean coast).

The *Fis* values, reflecting the heterozygote deficit within groups, did not exceed 0.161 (Mediterranean coast) except in the *A. m. mellifera* group, which had *Fis* = 0.418. Fisher's test was used to assess whether any of the groups could be considered as single populations. Genotypic differentiation showed 42 significant values out of 45 pair-wise comparisons. The Sardinia group was not significantly differentiated from any Italian group except for the North-east group. The North-west group was not significantly differentiated from the Buckfast breeding line.

The number of alleles and the observed and expected heterozygosity per locus and detailed allele frequencies are reported in Table III. Locus A107 was highly polymorphic with a total of 22 alleles detected (size range from 105 bp to 136 bp) and had the highest *Ho* values. Only five alleles were scored for locus A28 (130–140 bp) and loci A28 and A88 showed low heterozygosity values, in particular for the *A. m. ligustica* groups. Despite the low sample size, the Buckfast group displayed the highest *Ho* values in 3 out of 8 loci. Subspecies-specific alleles were scored for *A. m. mellifera* ($A14_{242}$, $Ap43_{131}$), *A. m. carnica* ($Ap43_{167}$) and *A. m. ligustica* ($A113_{222}$, $A14_{227}$, $Ap43_{149,151}$, $A7_{109,121,129,130}$). Both $A107_{138}$ and $A107_{142}$ were unique for the South and Mediterranean groups respectively. If we exclude Buckfast honeybees the number of *A.m. ligustica*-specific alleles doubles. The North-east group shows frequency similarity for many Carnica-characterizing alleles ($A113_{214}$, $A28_{136}$, $A14_{218,223}$, $A107_{134,136}$, $Ap43_{141}$, $Ab24_{103}$).

The gene diversity values varied in the range of 0.534 ± SE0.243 (Carnica) to 0.668 ± SE0.233 (Buckfast), with all Italian groups showing intermediate values.

The exact test for linkage disequilibrium at the 5% level for each pair of loci in each sample and overall did not provide any significant *P*-value after Bonferroni corrections.

Multilocus *Fst* values among the ten groups (Tab. IV) gave 31 significant pair-wise comparisons (12 of them highly significant), with Mellifera and Carnica showing significant comparisons in relation to all the other**.** The Mellifera group had the highest *Fst* scores which ranged between 0.207 (vs. South) and 0.322 (vs. Carnica). The Buckfast group shows intermediate values between Carnica and all the other *A. m. ligustica* groups, with the lowest scores detected for the Northern Italian groups (North-east and North-west). Pair-wise comparisons between *A. m. ligustica* groups scored values from 0.0009 to 0.0439: most of the comparisons with the Sardinia group were not significant. The last column of the same

**Table III.** Population data and detailed allele frequencies per locus per group. Allele size (in base pairs) is shown in the first column under the locus name. The gene number scored for each group is in the row beside each locus name. The number of alleles per locus (n) and the observed (*Ho*) and expected heterozygosities (*He*) are also reported.

| | Emilia Romagna | Carnica | Mellifera | Buckfast | North east | North west | Adriatic coast | Mediterr. coast | South | Sardinia |
|---|---|---|---|---|---|---|---|---|---|---|
| A113 | 44 | 37 | 11 | 15 | 38 | 28 | 30 | 71 | 28 | 20 |
| 200 | | | | | | | | 0.0070 | | |
| 202 | | | 0.1364 | | | | | | 0.0179 | |
| 210 | | | | | | 0.0179 | 0.0167 | 0.0070 | 0.0179 | |
| 214 | 0.3409 | 0.7432 | 0.0909 | 0.4000 | 0.4211 | 0.2321 | 0.2167 | 0.2042 | 0.2857 | 0.2750 |
| 216 | | | | | | 0.0179 | 0.0167 | | | 0.0250 |
| 218 | | | | | | | 0.0500 | 0.0423 | 0.0179 | |
| 220 | 0.5795 | 0.2432 | 0.6818 | 0.5000 | 0.5395 | 0.6786 | 0.6500 | 0.6761 | 0.6429 | 0.6000 |
| 222 | 0.0682 | | | | 0.0132 | 0.0179 | | 0.0423 | | 0.0250 |
| 224 | 0.0114 | | | 0.0333 | 0.0132 | | 0.0167 | | | |
| 226 | | | | | | | | | | 0.0500 |
| 228 | | 0.0135 | | 0.0333 | | 0.0179 | | 0.0141 | | |
| 230 | | | | 0.0333 | 0.0132 | 0.0179 | | | | 0.0250 |
| 238 | | | 0.0909 | | | | 0.0333 | 0.0070 | 0.0179 | |
| n = 13 | 4 | 3 | 4 | 5 | 5 | 7 | 7 | 8 | 6 | 6 |
| *Ho* | 0.4318 | 0.3514 | 0.0909 | 0.6000 | 0.4737 | 0.5000 | 0.5333 | 0.4648 | 0.3929 | 0.5500 |
| *He* | 0.5431 | 0.3882 | 0.5000 | 0.5867 | 0.5312 | 0.4841 | 0.5261 | 0.4973 | 0.5038 | 0.5600 |
| A28 | 44 | 53 | 13 | 14 | 38 | 30 | 37 | 73 | 29 | 22 |
| 130 | 0.0341 | 0.0943 | 0.9231 | 0.0714 | 0.0921 | 0.0500 | 0.0135 | 0.0205 | 0.0172 | |
| 132 | | | | | | | | | 0.0172 | |
| 136 | 0.9545 | 0.9057 | 0.0385 | 0.9286 | 0.8684 | 0.9167 | 0.9865 | 0.9384 | 0.9655 | 0.9545 |
| 138 | | | | | | 0.0167 | | | | |
| 140 | 0.0114 | | 0.0385 | | 0.0395 | 0.0167 | | 0.0411 | | 0.0455 |
| n = 5 | 3 | 2 | 3 | 2 | 3 | 4 | 2 | 3 | 3 | 2 |
| *Ho* | 0.0909 | 0.1132 | 0.1538 | 0.1429 | 0.2105 | 0.1667 | 0.0270 | 0.0685 | 0.0690 | 0.0000 |
| *He* | 0.0876 | 0.1709 | 0.1450 | 0.1327 | 0.2358 | 0.1567 | 0.0267 | 0.1174 | 0.0672 | 0.0868 |
| A14 | 42 | 52 | 13 | 11 | 28 | 30 | 35 | 64 | 30 | 20 |
| 216 | | | | | | 0.0167 | | | | |
| 218 | 0.6548 | 0.7885 | 0.2692 | 0.6364 | 0.7500 | 0.5667 | 0.5286 | 0.6641 | 0.5500 | 0.6500 |
| 220 | 0.0119 | 0.0385 | | 0.0909 | 0.0179 | 0.0833 | 0.0286 | 0.0313 | 0.0333 | |
| 221 | 0.0119 | 0.0192 | | | 0.0536 | 0.0167 | 0.0143 | 0.0156 | | |
| 223 | | 0.0288 | 0.1923 | 0.0909 | 0.0179 | 0.0167 | | | | 0.0500 |
| 224 | 0.0238 | 0.0192 | 0.0385 | | 0.0179 | 0.0167 | 0.0143 | 0.0156 | | |
| 226 | | 0.0096 | | | | | | | 0.0333 | |
| 227 | 0.0119 | | | | | 0.0333 | 0.0286 | | 0.0333 | |
| 228 | 0.0238 | | | 0.0455 | | | 0.0429 | 0.0078 | 0.0833 | |
| 229 | 0.0119 | | 0.3462 | 0.0455 | | | | | | |
| 232 | 0.0238 | | | 0.0455 | 0.0179 | 0.0167 | 0.0286 | 0.0313 | 0.0167 | 0.0250 |
| 234 | 0.2024 | 0.0385 | 0.0385 | 0.0455 | 0.1250 | 0.1500 | 0.2571 | 0.1563 | 0.1833 | 0.2750 |
| 236 | 0.0238 | 0.0577 | 0.0769 | | | 0.0833 | 0.0429 | 0.0703 | 0.0333 | |
| 238 | | | | | | | 0.0143 | 0.0078 | 0.0333 | |
| 242 | | | 0.0385 | | | | | | | |
| n = 15 | 10 | 8 | 7 | 7 | 7 | 10 | 10 | 9 | 9 | 4 |
| *Ho* | 0.4524 | 0.3654 | 0.3846 | 0.4545 | 0.5000 | 0.6667 | 0.6571 | 0.4688 | 0.5333 | 0.6000 |
| *He* | 0.5275 | 0.3704 | 0.7604 | 0.5702 | 0.4177 | 0.6400 | 0.6478 | 0.5271 | 0.6511 | 0.4988 |
| A107 | 41 | 48 | 11 | 13 | 33 | 21 | 35 | 62 | 30 | 10 |
| 134 | 0.0732 | | | 0.0769 | | 0.0952 | 0.0714 | 0.0242 | 0.0500 | 0.1500 |
| 136 | 0.0122 | | | 0.0769 | | 0.0238 | 0.0429 | 0.0161 | 0.0167 | |
| 138 | | | | | | | | | 0.0167 | |
| 142 | | | | | | | | 0.0081 | | |
| 146 | 0.0122 | 0.0313 | | 0.0385 | | | 0.0143 | 0.0403 | 0.0333 | 0.0500 |
| 148 | 0.1707 | 0.0313 | | 0.0385 | 0.0455 | 0.0476 | 0.1286 | 0.0806 | 0.0500 | 0.2000 |
| 150 | 0.0610 | 0.1667 | 0.0909 | | 0.1212 | 0.0952 | 0.0429 | 0.0403 | 0.0167 | 0.2000 |
| 152 | 0.0732 | 0.1563 | 0.0455 | 0.0769 | 0.0909 | 0.0476 | 0.0429 | 0.1129 | 0.1333 | 0.2000 |
| 154 | 0.0976 | 0.1250 | 0.0455 | 0.1923 | 0.0606 | 0.0476 | 0.1000 | 0.1129 | 0.1000 | 0.1000 |
| 156 | 0.1463 | 0.0729 | 0.1818 | | 0.1970 | 0.1190 | 0.1714 | 0.2097 | 0.1000 | 0.1000 |
| 158 | 0.0610 | 0.0833 | 0.1818 | 0.2308 | 0.0909 | 0.1667 | 0.0571 | 0.1048 | 0.2333 | |
| 160 | 0.0366 | 0.0417 | 0.0909 | 0.0769 | 0.0606 | 0.0476 | 0.0286 | 0.0806 | 0.1167 | |
| 162 | 0.0366 | 0.1563 | 0.2727 | 0.1154 | 0.1061 | 0.0476 | 0.0429 | 0.0726 | 0.0167 | |
| 164 | 0.1220 | 0.0729 | | 0.0385 | 0.1364 | 0.1429 | 0.1143 | 0.0565 | 0.0500 | |
| 166 | 0.0366 | 0.0521 | 0.0909 | | 0.0606 | 0.0238 | 0.0429 | | | |
| 168 | 0.0488 | 0.0104 | | | 0.0303 | 0.0714 | 0.1000 | 0.0242 | 0.0500 | |
| 172 | 0.0122 | | | 0.0385 | | 0.0238 | | 0.0161 | 0.0167 | |
| n = 17 | 15 | 12 | 8 | 11 | 11 | 14 | 14 | 15 | 15 | 7 |
| *Ho* | 0.7805 | 0.7917 | 0.7273 | 0.7692 | 0.8788 | 0.8571 | 0.8286 | 0.8548 | 0.7000 | 0.8000 |
| *He* | 0.9001 | 0.8837 | 0.8306 | 0.8669 | 0.8861 | 0.9014 | 0.9024 | 0.8931 | 0.8817 | 0.8350 |

**Table III.** Continued.

| | Emilia Romagna | Carnica | Mellifera | Buckfast | North east | North west | Adriatic coast | Mediterr. coast | South | Sardinia |
|---|---|---|---|---|---|---|---|---|---|---|
| A(B)24 | 38 | 55 | 13 | 13 | 34 | 30 | 29 | 62 | 24 | 19 |
| 93 | | | | | 0.0147 | | | 0.0081 | | |
| 95 | | | 0.1923 | | 0.0294 | 0.0667 | | 0.0081 | | |
| 97 | 0.1944 | 0.1000 | 0.6538 | 0.1923 | 0.1176 | 0.1333 | 0.2069 | 0.2661 | 0.2917 | 0.1842 |
| 103 | | 0.0364 | | 0.0385 | 0.0294 | | 0.0345 | | | |
| 105 | 0.2500 | 0.3818 | 0.1154 | 0.3077 | 0.2353 | 0.2500 | 0.1724 | 0.1290 | 0.1458 | 0.2105 |
| 107 | 0.5556 | 0.4818 | 0.0385 | 0.4615 | 0.5735 | 0.5500 | 0.5690 | 0.5887 | 0.5417 | 0.6053 |
| 109 | | | | | | | 0.0172 | | 0.0208 | |
| n = 7 | 3 | 4 | 4 | 4 | 6 | 4 | 5 | 5 | 4 | 3 |
| *Ho* | 0.5556 | 0.5636 | 0.2308 | 0.3846 | 0.5882 | 0.4667 | 0.5517 | 0.5968 | 0.6667 | 0.5263 |
| *He* | 0.5910 | 0.6107 | 0.5207 | 0.6538 | 0.5999 | 0.6128 | 0.6023 | 0.5658 | 0.5998 | 0.5554 |
| A88 | 42 | 45 | 8 | 10 | 29 | 25 | 32 | 72 | 24 | 20 |
| 136 | | 0.0222 | | 0.0500 | | | | | | |
| 141 | | 0.0111 | 0.0625 | 0.0500 | 0.0172 | | 0.0625 | 0.0069 | 0.0417 | 0.0750 |
| 144 | 0.0476 | 0.0333 | 0.6250 | 0.1000 | 0.0862 | 0.0800 | 0.0156 | 0.0486 | 0.0208 | |
| 148 | | 0.0111 | 0.1250 | 0.1000 | | | 0.0156 | | | |
| 150 | 0.0119 | 0.0667 | | 0.1000 | 0.0862 | 0.1000 | 0.0625 | 0.0694 | | 0.0500 |
| 151 | | | | 0.0500 | | | | 0.0069 | | |
| 152 | 0.7857 | 0.8333 | 0.1875 | 0.5000 | 0.7414 | 0.6800 | 0.7969 | 0.7569 | 0.9167 | 0.8750 |
| 153 | 0.0595 | | | 0.0500 | | 0.0400 | 0.0469 | 0.0625 | | |
| 154 | 0.0952 | 0.0222 | | | 0.0690 | 0.1000 | | 0.0486 | 0.0208 | |
| n = 9 | 5 | 7 | 4 | 8 | 5 | 5 | 6 | 7 | 4 | 3 |
| *Ho* | 0.2619 | 0.2889 | 0.3750 | 0.9000 | 0.3448 | 0.2400 | 0.2813 | 0.3333 | 0.1667 | 0.2000 |
| *He* | 0.3676 | 0.2988 | 0.5547 | 0.7100 | 0.4304 | 0.5096 | 0.3545 | 0.4135 | 0.1571 | 0.2262 |
| Ap43 | 45 | 52 | 13 | 13 | 29 | 30 | 39 | 71 | 28 | 18 |
| 131 | | | 0.0769 | | | | | | | |
| 133 | | 0.0288 | 0.2308 | 0.0385 | | 0.0167 | | | 0.0179 | |
| 135 | 0.0111 | 0.1923 | 0.4615 | 0.3077 | 0.1724 | 0.0500 | 0.1026 | 0.0563 | 0.0714 | 0.1944 |
| 137 | 0.0111 | | 0.0385 | | | 0.0167 | 0.0256 | 0.0282 | | 0.0278 |
| 139 | 0.0111 | | | 0.0385 | | 0.0167 | | | | 0.0278 |
| 141 | 0.0556 | 0.2788 | 0.0385 | 0.0769 | 0.2069 | 0.1833 | 0.0385 | 0.0915 | 0.0536 | 0.0556 |
| 143 | 0.2889 | 0.4038 | 0.0769 | 0.4615 | 0.5345 | 0.4500 | 0.4487 | 0.6197 | 0.6250 | 0.3889 |
| 145 | 0.4778 | 0.0769 | 0.0769 | 0.0769 | 0.0690 | 0.1500 | 0.3077 | 0.1127 | 0.1964 | 0.1944 |
| 147 | 0.0889 | 0.0096 | | | 0.0172 | 0.1167 | 0.0641 | 0.0423 | 0.0357 | 0.1111 |
| 149 | 0.0444 | | | | | | 0.0128 | 0.0211 | | |
| 151 | 0.0111 | | | | | | | 0.0211 | | |
| 155 | | | | | | | | 0.0070 | | |
| 167 | | 0.0096 | | | | | | | | |
| n = 13 | 9 | 7 | 7 | 6 | 5 | 8 | 7 | 9 | 6 | 7 |
| *Ho* | 0.5778 | 0.5769 | 0.5385 | 0.6923 | 0.4138 | 0.8000 | 0.4103 | 0.4366 | 0.5000 | 0.6111 |
| *He* | 0.6748 | 0.7152 | 0.7130 | 0.6775 | 0.6367 | 0.7244 | 0.6870 | 0.5882 | 0.5612 | 0.7562 |
| A7 | 36 | 34 | 9 | 10 | 29 | 20 | 33 | 68 | 28 | 14 |
| 105 | 0.0139 | | | 0.0500 | | | | 0.0074 | | 0.0357 |
| 107 | 0.1111 | 0.0294 | | | 0.0172 | | 0.1667 | 0.0368 | 0.0179 | 0.0357 |
| 108 | 0.0139 | | | 0.0500 | 0.0517 | 0.0750 | 0.0152 | 0.0441 | 0.1607 | 0.0714 |
| 109 | | | | | 0.0345 | 0.0250 | 0.0303 | 0.0662 | | 0.0357 |
| 111 | 0.0833 | 0.0147 | 0.1667 | | 0.0172 | 0.0750 | 0.0909 | 0.0074 | 0.0179 | 0.0357 |
| 112 | 0.0278 | 0.0588 | | 0.0500 | 0.1034 | | | 0.0441 | | |
| 113 | | 0.1324 | 0.2500 | 0.0500 | 0.0172 | 0.1750 | 0.0303 | 0.0515 | | |
| 115 | | 0.0441 | | | | 0.0500 | | | 0.0179 | |
| 116 | 0.1111 | | | 0.1000 | 0.0172 | 0.0250 | 0.0909 | 0.0294 | | 0.0357 |
| 118 | 0.3750 | 0.3676 | | 0.1500 | 0.3276 | 0.2500 | 0.3485 | 0.1838 | 0.1250 | 0.3571 |
| 119 | 0.0139 | | | 0.0500 | 0.0517 | | 0.0303 | 0.0294 | | |
| 120 | 0.0694 | 0.2059 | 0.0833 | 0.1500 | 0.1897 | 0.1250 | 0.0909 | 0.1765 | 0.3750 | 0.1786 |
| 121 | 0.0278 | | | | | | | 0.0074 | 0.0179 | |
| 122 | 0.0417 | 0.1176 | 0.3333 | 0.0500 | 0.0690 | 0.0750 | 0.0909 | 0.1618 | 0.1607 | 0.1071 |
| 123 | | | | 0.0500 | | 0.0250 | | 0.0147 | | 0.0357 |
| 124 | 0.0139 | | | 0.1500 | 0.0862 | | | 0.0588 | 0.0536 | 0.0357 |
| 126 | | | | | | | | 0.0294 | | 0.0357 |
| 128 | 0.0278 | 0.0147 | | | | | | | 0.0179 | |
| 129 | 0.0556 | | | | | | 0.0152 | 0.0074 | | |
| 130 | 0.0139 | | | | 0.0172 | 0.1000 | | 0.0368 | 0.0357 | |
| 131 | | | | 0.0500 | | | | 0.0074 | | |
| 136 | | 0.0147 | 0.1667 | 0.0500 | | | | | | |
| n = 22 | 15 | 10 | 5 | 13 | 13 | 11 | 11 | 19 | 11 | 12 |
| *Ho* | 0.8056 | 0.7059 | 0.5000 | 0.9000 | 0.8621 | 0.7500 | 0.6061 | 0.8088 | 0.6429 | 0.7857 |
| *He* | 0.8148 | 0.7842 | 0.7639 | 0.9000 | 0.8258 | 0.8600 | 0.8145 | 0.8887 | 0.7864 | 0.8138 |

**Table IV.** Multilocus *Fst* values among 10 groups (upper right matrix) and significance *P*-values (lower left matrix). ***: $P < 0.01$; *: $0.01 < P < 0.05$; n.s.: $P > 0.05$). The additional grey column shows comparisons of Italian samples (pooled into a single group called Ligustica) with other taxa.

| | Emilia Romagna | Carnica | Mellifera | Buckfast | North east | North west | Adriatic coast | Mediterr. coast | South | Sardinia | Ligustica |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Emilia Romagna | | 0.0698 | 0.2959 | 0.0406 | 0.0305 | 0.0179 | 0.0009 | 0.0351 | 0.0439 | 0.0067 | |
| Carnica | * | | 0.3221 | 0.0394 | 0.0239 | 0.0611 | 0.0819 | 0.0790 | 0.0842 | 0.0551 | 0.0580* |
| Mellifera | *** | *** | | 0.2074 | 0.2721 | 0.2444 | 0.2838 | 0.2833 | 0.3007 | 0.2940 | 0.2805*** |
| Buckfast | * | ns | * | | 0.0081 | 0.0087 | 0.0302 | 0.0234 | 0.0303 | 0.0243 | 0.0184ns |
| North east | *** | * | * | ns | | 0.0074 | 0.0235 | 0.0130 | 0.0287 | 0.0114 | |
| North west | ns | * | * | ns | ns | | 0.0060 | 0.0072 | 0.0191 | 0.0094 | |
| Adriatic coast | ns | *** | *** | * | *** | * | | 0.0131 | 0.0212 | –0.0026 | |
| Mediterranean coast | *** | *** | * | ns | * | ns | *** | | 0.0075 | 0.0128 | |
| South | *** | *** | * | ns | * | * | * | * | | 0.0174 | |
| Sardinia | ns | * | *** | * | * | ns | ns | ns | ns | | |

table shows *Fst* values among the four taxa of the present study, where all *A. m. ligustica* samples are pooled into the group Ligustica. Values computed using Goldstein's distance showed the same pattern (data not shown).

Mellifera was the outgroup chosen for Neighbor Joining analyses (trees not shown): different genetic distance methods returned the same tree topology, with Carnica placed basal to a clade including all *A. m. ligustica* groups, while Buckfast and Mellifera were sister taxa. Within the *A. m. ligustica* clade, the North-east and North-west groups were the most external and showed the highest bootstrap values.

Based on the analysis of whole data set using 'Structure', five was inferred as the most likely number of population (*k*): both Mellifera and Carnica groups had a high proportion of membership respectively in only one of these inferred populations, while most *A. m. ligustica* samples shared their proportion of membership in the remaining three inferred populations, not following a clear geographic structure. Buckfast, North-east and North-west groups showed a lower proportion of membership compared to the remaining Italian groups. The analysis of the second dataset allowed us to assign 4 out of 15 honeybees from the artificial Buckfast breeding line (3 to Carnica and 1 to the Emilia-Romagna group), while the individual analysis of North-east honeybees showed for most of them a high proportion of membership in the same population that clustered Carnica honeybees (Fig. 2).

## 4. DISCUSSION

Microsatellites confirmed their discriminating power for closely related taxa: a clean distinction could be made not only between honeybees belonging to different evolutionary branches, but also between *A. m. carnica* and *A. m. ligustica* honeybees (both belonging to branch C), for which loci A14, A113 and Ap43 showed to be the most informative. Locus Ap43 revealed the presence of almost one private allele for each of the three taxa.

Moreover, most of the samples from the Buckfast breeding line could not be correctly assigned by individual analysis to any of the three subspecies included in the reference database, thus confirming their hybrid origin. The high mean number of alleles detected (despite the low sample size) is therefore not surprising and further underscores the genetic diversity in this artificial lineage.

The suite of microsatellites we used provided an objective diagnostic method for confirming the origin of *A. m. ligustica* breeding lines and detecting both hybrids and the potential introgression of other subspecies. Cluster analysis revealed the presence of non-*A. m. ligustica* alleles in the northern Italian groups. Particularly in the North-east group, Carnica-characterizing alleles were identified. The individual analyses showed evidence of introgression not only in samples from the known natural hybridization zone (Friuli), but also in samples taken from honeybee colonies in the north of the Veneto region; this might be due

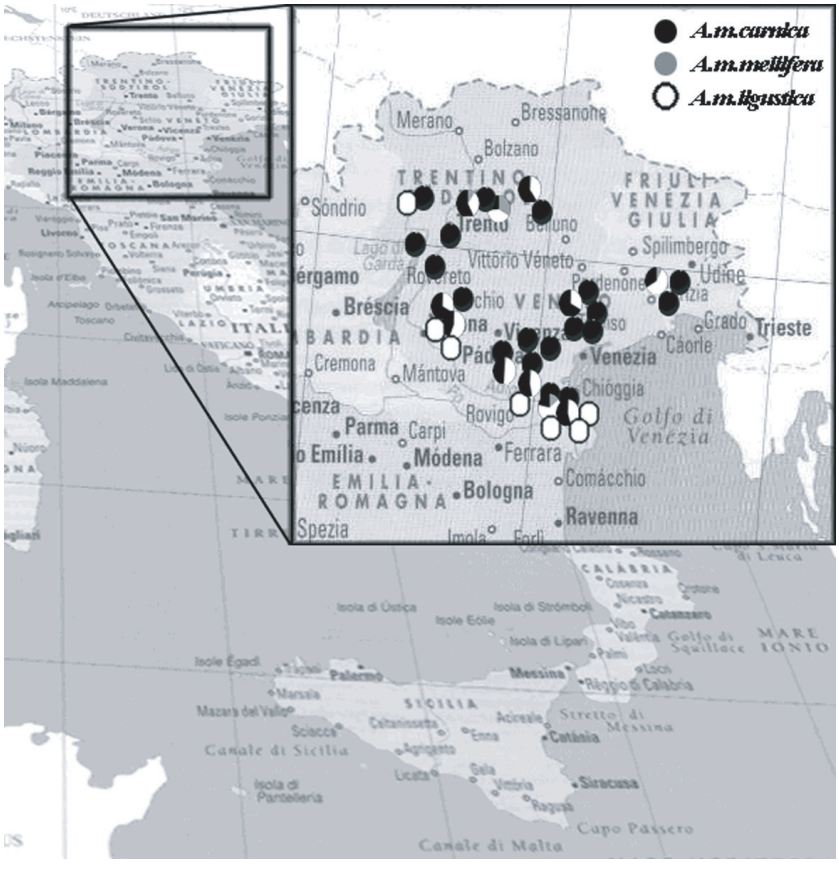


**Figure 2.** Detail of the individual analysis of samples from the North-east group. *A. m. carnica* alleles are largely present in the Veneto region.

to either or both natural migration and transport by beekeepers.

Professional beekeeping practices can interfere with attempts to conserve indigenous honeybee subspecies. Controlled and co-ordinated breeding programmes in Italy, which involve the intensive rearing of thousands of queens produced by a few selected mothers, have resulted in the loss of genetic diversity within native populations (Bolchi Serini et al., 1983; Marletto et al., 1984).

In a study by Franck et al. (2000), genetic structure was detected despite the fact that the number of alleles per locus was substantially smaller than in the present study (e.g. 5 fewer alleles for locus A113). According to the data presented herein, none of the Italian groups shows a population substructure and local genetic variability is as great as genetic variability throughout the peninsula, as may be seen from the non-significant *Fst* values and poor resolution of the inner nodes of the NJ-trees. The higher number of alleles detected may thus only mirror the effect of the dense coverage of the sampling area.

The population of Sardinia may be symptomatic. In spite of the insular situation, both the Fisher and *Fst* methods returned non-significant differences with the other *A. m. ligustica* groups, thus revealing the absence of genetic isolation. This mirrors the effects of the yearly large-scale importation of queens and honeybees from the Italian peninsula (Floris and Prota, 1994, Verardi et al., 1998).

The conclusions that may be drawn from the present study indicate that Italian

honeybees still have unique genetic characteristics typical of the subspecies *A. m. ligustica,* which deserve to be conserved. In light of the potential introgression of genes from artificial breeding lines and adjacent subspecies in Northern Italy, it seems warranted to carefully plan and continue breeding efforts to maintain *A. m. ligustica* as a subspecies serving the beekeeping industry in Italy. As Italian environments show huge differences across the peninsula and islands, we were surprised not to have found specific ecotypes within *A. m. ligustica*. Given the population structure, also as evidenced by individual analysis, it currently looks as if the queen breeding routine – with queen rearing in the north, but queen mating in the south – has caused an amalgamation of local populations into one large Italian population. We recommend that the study of this suite of microsatellites and particularly of the highly polymorphic loci A7 and A107 be continuously monitored through population studies in small geographic areas to detect any changes in the population structure.

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**Caractérisation génétique des abeilles italiennes, *Apis mellifera ligustica*, par les polymorphismes de l'ADN microsatellite.**

***Apis mellifera ligustica* / microsatellite / variabilité génétique / génétique des populations / conservation / diversité biologique / introgression / Italie**

**Zusammenfassung – Genetische Charakterisierung der italienischen Honigbiene, *Apis mellifera ligustica*, mittels Mikrosatelliten-DNA-Polymorphismen.** Wir erfassten die genetische Variabilität von Honigbienen der Rasse *Apis mellifera ligustica* über ganz Italien hinweg und in Sardinien. Die Genotypen zweier anderer Unterarten (*Apis mellifera mellifera*, *Apis mellifera carnica*) und Proben der künstlichen Buckfast-Zuchtlinie wurden zum Vergleich ebenfalls untersucht. Insgesamt wurden über vier Jahre hinweg (2001–2004) Proben aus 379 Völkern von verschiedenen Standorten genommen. Alle *A. m. ligustica* Proben wurden sieben verschiedenen regionalen Gruppen zugeordnet (Abb. 1). Genetische Polymorphismen wurden für acht Mikrosatellitenloci mittels automatisierter Kapillarelektrophorese untersucht (Locus-spezifische PCR-Bedingungen sind in Tab. I zusammengestellt). Die Mikrosatellitendaten der zehn Gruppen ermöglichte die Erfassung der allgemeinen Populationsparameter (Tab. II), die Darstellung der phylogenetischen Beziehungen zwischen den Gruppen und deren jeweilige Populationsstruktur, ebenso wie die Erstellung individueller Analysen.
Die in dieser Studie verwendeten genetischen Marker belegen das Potential der Mikrosatelliten-DNA-Loci für die Unterscheidung eng verwandter Taxa, da es möglich war, nicht nur *A. m. mellifera*-Proben (dem Evolutionszweigs M zugeordnet) von anderen zu unterscheiden, sondern auch zwischen *A. m. carnica* and *A. m. ligustica* (beide zum gleichen geographischen Zweig C gehörend) zu differenzieren. Die meisten Loci hatten unterartspezifische Allele (Tab. III), wobei der Locus Ap43 ein jeweils exklusives Allel für jedes der drei Taxa aufwies. Die Analyse der künstlichen Buckfast-Zuchtlinie belegte deren Ursprung als Hybrid und deren hohe genetische Diversität. Unser Genotypisierungsansatz erlaubte zusätzlich die Detektion von Hybridindividuen in Bienenproben aus dem Nordosten Italiens (Abb. 2).
Phylogenetische und individuelle Analysen belegten, dass *A. m. ligustica* inzwischen eine grosse Population darstellt, wahrscheinlich als Ergebnis einer intensiven Bienenhaltung, insbesondere durch die Wanderung von Bienenvölkern und den grossangelegten kommerziellen Austausch von Königinnen. Die Ergebnisse unserer Untersuchung stellen ein Datenset dar, das künftigen Programmen zur lokalen Konservierung der Biodiversität und auch kontrollierten Zuchtprogrammen von Nutzen sein kann. Die Mikrosatelliten-DNA-Analyse ist ein effizientes Verfahren, um die Introgression fremder Allele in natürlichen und kommerziell genutzten *A. m. ligustica*-Populationen zu entdecken und zu verfolgen.

***A. m. ligustica* / Mikrosatellit / genetische Variabilität / Konservierung**

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