Journal of Apicultural Research 50(1): 51-84 (2011)
DOI 10.3896/IBRA.1.50.1.06

REVIEW ARTICLE

A review of methods for discrimination of honey bee populations as applied to European beekeeping

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Received 5 May 2010, accepted subject to revision 30 August 2010, accepted for publication 24 November 2010.

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Summary

Here, scientists from 19 European countries, most of them collaborating in Working Group 4: "Diversity and Vitality" of COST Action FA 0803 "Prevention of honey bee COlony LOSSes" (COLOSS), review the methodology applied in each country for discriminating between honey bee populations. Morphometric analyses (classical and geometric) and different molecular markers have been applied. Even if the approach has been similar, however, different methodologies regarding measurements, landmarks or molecular markers may have been used, as well as different statistical procedures. There is therefore the necessity to establish common methods in all countries in order to have results that can be directly compared. This is one of the goals of WG4 of the COLOSS project.

Revisión de los métodos para discriminar las poblaciones de abejas aplicados en la apicultura europea

Resumen

Científicos de 19 países europeos, la mayoría de ellos colaboradores del Grupo de Trabajo 4: "Diversidad y Vitalidad" de la Acción COST FA 0803 "Prevención de la mortalidad de colmenas" (COLOSS), revisan aquí la metodología aplicada en cada país para discriminar a las poblaciones de abejas. Se han aplicado análisis morfométricos (clásico y geométrico) y distintos marcadores moleculares. Incluso si el enfoque ha sido el mismo, se han usado diferentes metodologías en relación a las medidas, puntos de referencia o marcadores moleculares, así como diferentes procedimientos estadísticos. Por lo tanto, existe la necesidad de establecer métodos comunes en todos los países para tener resultados que puedan ser directamente comparados. Este es uno de los objetivos del GT4 del proyecto COLOSS.

Keywords: honey bee, beekeeping, sub-species, morphometrics, DNA, isoenzymes

Introduction

Traditionally, the intraspecific taxonomy of the honey bee *Apis mellifera* L. has been based on morphology. At present, about 26 sub-species of *A. mellifera* are recognized on the basis of morphometric characters (Ruttner, 1988, 1992; Sheppard *et al.*, 1997; Engel, 1999; Sheppard and Meixner, 2003). Those sub-species are also described as "geographic sub-species" since their distributions correspond to distinct geographic areas.

Five evolutionary lineages have been characterized based on morphometric, molecular, ecological, ethological and physiological traits (reviewed by De la Rúa *et al.*, 2005). Four of these occur naturally in the Mediterranean Basin: the African lineage (A); the West and North European lineage (M); the Southeast Europe lineage (C); and the Near and Middle Eastern lineage (O). Beekeeping manipulations such as commercial breeding and migratory beekeeping have, however, affected the genetic variability of local honey bee populations.

In this paper, scientists from 19 European countries, most of them are collaborating in Work Group 4: "Diversity and Vitality" of COST Action FA 0803: "Prevention of honey bee COlony LOSSes" (COLOSS) (Neumann and Carreck, 2010) review the methodology applied in each country for discriminating honey bee populations. Since the beginning of the 20th century, commercial bee breeding has been dominated by introduced honey bees. As a consequence of this direct replacement and gene flow between native and commercial honey bee populations, native populations have been considered to be extinct in many parts of Europe (Jensen *et al.*, 2005). In many countries there is a kind of "certification" for local honey bee strains. This information will be useful for conservation and selection purposes, both a major goal and a necessity as honey bees decline with unforeseen consequences all over the world.

Countries

Bulgaria

According to Ruttner's morphometric analysis (1988), the sub-species *A. m. macedonica* occurs in Bulgaria. An alternative view is the existence in Bulgaria of a native local honey bee *A. m. rodopica* (Petrov, 1990) which is of type "*carnica*" in a broad sense (Engel, 1999).

Local Bulgarian honey bees have been studied using classical morphometric analysis since 1935 (Lazarov, 1935, 1936; Tzonev, 1960). The data obtained were used for organization of selection programmes in Bulgaria. During 1971-1990 the selection was carried out in two directions: thoroughbred selection of the local honey bee, and inter-sub-species hybridization (Velichkov, 1970). In the past, for more than three decades, *A. m. ligustica, A. m. carnica* and *A. m. caucasica* were reared in Bulgaria. The local Bulgarian bee was threatened by many activities including queen breeding (which reduces the effective population size) and importation of foreign queens (which modifies local bees through hybridization). All of these activities have had an impact on the genetic variability of the honey bees of the country.

The local Bulgarian honey bee, which is productive and well adapted to the local conditions is now a basis for selective work in the country. A morpho-ethological analysis using specific characteristics has been carried out in order to determine the sub-species of local bees (Petrov, 1990, 1991, 1993a, b, 1995, 1997a, b, c, 2000; Petrov and Hristov, 2006; Petrov and Yankov, 2006; Petrov *et al.,* 2006). Biochemical and genetic research on the polymorphism of some isoenzymic systems has also been carried out during the last 15 years (Ivanova *et al.*, 2004; Ivanova and Staykova, 2005; Ivanova *et al.*, 2006, 2007; Ivanova *et al.*, 2008). Different methods of DNA analyses have been fragmentarily applied in this direction (Ivanova *et al.*, 2004; Ivanova *et al.*, 2008). The usage of molecular markers such as mtDNA, RAPD, and microsatellite DNA is a very important part of the complex of methods planned for studying the discrimination of honey bee populations in Bulgaria.

Morphometrical, ethological, isoenzymic and DNA analysis have been purposefully used to clarify the sub-species standard of the local honey bee, which is a part of the European genetic resource of *A. mellifera*, productive enough and well adapted to the specific conditions for the country (Petrov and Ivanova, 2009; Ivanova and Bouga, 2009; Ivanova *et al*., 2010). The following are the parameters used for the discrimination of Bulgarian honey bee populations:-

Classical morphometrics (Alpatov, 1945, 1948a) and routine activities

The following measurements are being performed: 1. for queens: weight of a non-inseminated queen (mg), weight of an inseminated queen (mg), length of proboscis (mm), forewing length (mm), forewing width (mm), cubital index, discoidal shifting (%), tarsal index (%), diameter of the spermatheca (mm), colour of the abdomen; 2. for drones: weight (mg), length of proboscis (mm), forewing length (mm), forewing width (mm), cubital index, discoidal shift (%), sum of the length of 3rd and 4th abdominal tergum (mm), coloration of the abdominal terga, colour of the thorax; 3. for workers: weight (mg), length of proboscis (mm), length and width of the forewing (mm), cubital index, discoidal shifting (%), wax-mirror back border (%), length of the 5th abdominal tergum (mm), hair index of the 4th abdominal tergum, sum of the length of 3rd and 4th abdominal terga (mm), length of the hind leg (mm), tarsal index (%), coloration of the abdominal terga, colour of the 2nd abdominal tergum, colour of the 3rd to 6th abdominal terga.

Bees from more than 500 mountainous and plain micro regions of the country have now been studied. Over 15,700 samples have been collected from worker bees and drones for morphometric analysis, and 921,590 microscopic measurements have been performed. Some differences between local Bulgarian bees and other sub-species of *A. mellifera* were reported by Petrov (1996) and Petrov and Petkova (1996, 1997). Comparing the length of the hind leg, tarsal index, forewing length and width, and sum of the length of 3rd and 4th abdominal terga of Bulgarian worker bees, it seems that local honey bees are reliably different ($P \leq 0.05$; 0.01; 0.001) from *A. m. carnica*, *A. m. carpatica*, *A. m. ligustica*, *A. m. mellifera*, *A. m. caucasica* and *A. m. anatoliaca*. The measurements of the morphometrical characteristics noted above for Bulgarian honey bees are as follows: length of the hind leg: 8.091 ± 0.026; tarsal index: 56.125 ± 0.248; forewing length: 9.102 ± 0.02; forewing width: 3.222 ± 0.006 and sum of the length of 3rd and 4th abdominal terga: 4.582 ± 0.009.

Biochemical markers

More than 5000 worker bees have been studied using PAGE and Starch GE (Davis, 1964; Smithies, 1955) during the period 1991 – 2008. Isoenzymic systems and loci were found to be polymorphic in Bulgarian honey bees: *MDH-1* (three alleles: MDH^{65}, MDH^{80} and MDH^{100}), *ME* (four alleles: ME^{90}, ME^{100}, ME^{106} and ME^{115}), *EST-3* (six alleles: (EST^{80}, EST^{88}, EST^{94}, EST^{100} EST^{105} and EST^{118}), *ALP* (three alleles: ALP^{80}, ALP^{90} and ALP^{100}), *PGM* (four alleles: PGM^{80}, PGM^{100}, PGM^{114} and PGM^{125}) and *HK* (three alleles: HK^{87}, HK^{100} and HK^{110}). Data on isoenzymic polymorphisms detected in local Bulgarian bees, which are now a base for selective work in Bulgaria are presented in Table 1.

Table 1. Polymorphic loci, alleles and allele frequencies in local Bulgarian honey bees.

Polymorphic locus	Alleles	Allele frequency Local honey bees
MDH-1	MDH^{65}	0.410 – 0.213
	MDH^{100}	0.590 – 0.787
ME	ME^{90}	0.0 – 0.019
	ME^{100}	0.738 – 0.955
	ME^{106}	0.243 – 0.045
EST-3	EST^{80}	0.0 – 0.082
	EST^{88}	0.0 – 0.043
	EST^{94}	0.0 – 0.008
	EST^{100}	1.0 – 0.918
	EST^{118}	0.0 – 0.026
PGM	PGM^{100}	0.890 – 0.942
	PGM^{114}	0.058 – 0.110
HK	HK^{87}	0.0 – 0.063
	HK^{100}	1.0 – 0.909
	HK^{110}	0.0 – 0.028
ALP	ALP^{80}	0.518 – 0.700
	ALP^{100}	0.482 – 0.300

Comparing allozyme data for Bulgarian honey bees with other data for *A. m. macedonica* and *A. m. carnica* concerning *MDH-1*, *EST-3*, *ME*, *ALP, PGM* and *HK* polymorphism (Dedej *et al.,* 1996; Bouga *et al*., 2005a; Kandemir *et al*., 2005), it can be concluded that Bulgarian honey bees are genetically much closer to *A. m. macedonica* than to *A. m. carnica*.

Croatia

According to Ruttner (1988), bees from Croatia are *A. m. carnica*. The bees are large with dark body colour, broad and dense tomenta, short grey cover hair and recognizable morphometric characteristic (e.g., high value of cubital index). Carniolan bees are well adapted to local climatic and foraging conditions (Ruttner, 1988; Bubalo *et al*., 2002). Croatia has three distinctive climatic and foraging zones: the Pannonian plains in the north (with a continental climate); the central mountain region and highlands with sub-alpine climate (with longer and severe winters); and coastal regions and islands with a Mediterranean climate. The importation of honey bee sub-species other than *A. m. carnica* has been legally banned since the establishment of Croatia. For centuries, there was no trade or imports

of bees except for an exchange of bees between Slovenia and Croatia, both of which have Carniolan bees.

Discrimination of subpopulations in Croatia has been performed using morphometric and biological traits. Strains from northern continental plains and the central mountain region have similar annual population cycles, swarming tendencies and wintering ability, typical for Carniolan bees. The bees from the coast, however, especially from southern Dalmatia, show a Mediterranean type of brood rearing phenology (Bubalo *et al.*, 1997; Kezić *et al.*, 2001).

Morphometric studies

Studies of morphometric characteristics and colony traits intensified during the last two decades. The majority of studies used wing size and cubital index (Duvančić *et al.*, 1996; Dražić *et al.*, 2003). One study included measurements of leg segments for comparison of bees originating in three climatic Croatian regions (Dražić *et al.*, 1998, 1999). Results were similar to those given by Ruttner (1988). Based on wing features (length, width, and venation) it is possible to discriminate subpopulations of bees from distinctive Croatian regions (Table 2). Segments of the hind leg (length of femur, tibia and metatarsus, width of tibia and metatarsus and surface area of tibia and metatarsus) were measured for three populations of bees originating from the Pannonian, mountain and Mediterranean regions (Dražić *et al.*, 1998, 1999).

Principal component analysis (Fig. 1) indicates that Factor 1 accounts for about 19.68% of the total variability and includes differences in width and surface area of the tibia and metatarsus. Factor 2 comprising 11.43% of the total variability, is composed mainly of characteristics of lengths of leg segments. Based on morphometrical measures of leg segments it is possible to distinguish Mediterranean from other populations of Croatian bees. The differences between Pannonian and mountain strains are not so distinct.

Table 2. Morphometric characteristics of *A. m. carnica* sub-populations reported by Ruttner (1988) and by Dražić *et al.* (1998, 1999).

Population	N	Forewing length	SD	Reference
Alpine (Austria)	21	9.403	0.149	Ruttner, 1988
Pannonian (Hungary)	16	9.265	0.160	
Dalmatia (Croatia)	6	9.177	0.151	
Croatia mountain region	209	9.263	0.231	Dražić *et al.* 1998, 1999
Croatia Pannonian region	210	9.198	0.196	
Croatia Mediterranean region	210	9.157	0.233	

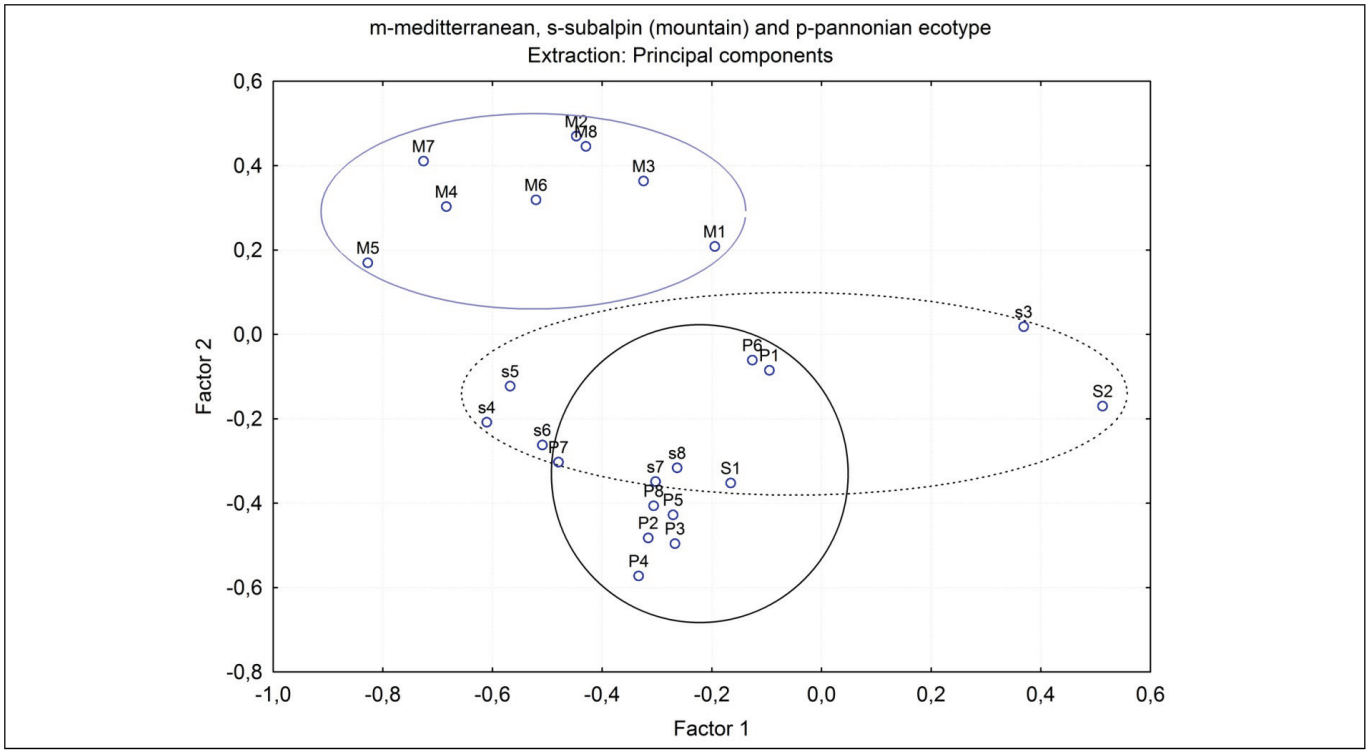

Fig. 1. Factor analysis of length, width and surface of the hind leg segments per ecotype of honey bees in Croatia.

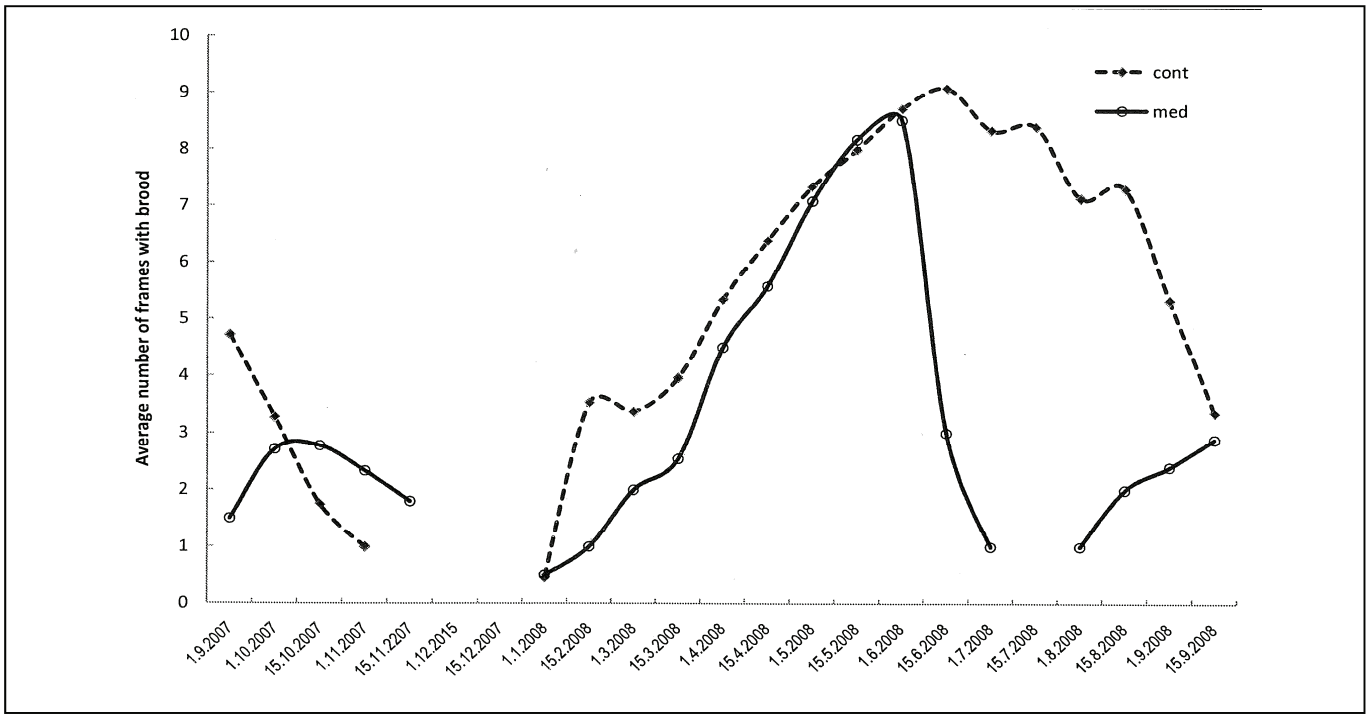

Fig. 2. Brood cycle of colonies from continental and Mediterranean region in Croatia .

Table 3. Average number and standard deviation (SD) of worker brood cells for Slovenian, Croatian and Austrian colonies at Lunz am See, Austria (alpine climate) and Mala Dapčevica, Croatia (continental) and from 14 March to 18 August 1993.

Location of experimental colonies	Origin of colonies	Average no of worker brood cells	SD
Lunz am See	Slovenia	129,328	35,161
	Croatia	123,802	53,556
	Austria	131,944	25,591
Mala Dapčevica	Slovenia	203,048	52,179
	Croatia	235,676	26,806
	Austria	209,962	50,434

Biological traits

Biological traits of different Carniolan honey bee strains were analysed simultaneously in continental (Mala Dapčevica, Croatia) and alpine (Lunz am See, Austria) climates (Bubalo *et al.,* 1994; Kezić *et al.,* 1994; Bubalo *et al.*, 1997, 2002). Colonies showed significant differences in number of unsealed and sealed worker brood cells in both climates. In the whole season, there was no significant difference between strains regarding development of drone brood. In the continental climate, the number of eggs laid was greater for all strains (Table 3).

Brood rearing cycles of native Pannonian and Mediterranean strains in Croatia are shown on Fig. 2. In the continental region, the broodless period lasts from the end of October to the middle of February. In years with mild winters, the broodless periods can be shorter. Spring build up is rapid. During summer, brood production depends on the availability of nectar and pollen. Colonies in the coastal Mediterranean region usually have two broodless periods; one short period during winter and a second period during the warmest part of the summer.

Molecular analysis

Studies using molecular analysis confirmed the genetical similarity of Croatian and Slovenian honey bee populations (Sušnik *et al.*, 2004). Samples collected in coastal Croatia belong to the Central Mediterranean and Southeast European evolutionary C-lineage (Muñoz *et al.*, 2009).

Denmark

In Denmark, the original local sub-species *A. m. mellifera* today only occurs on the Island of Læso, where a few beekeepers maintain the population. In the rest of Denmark, beekeepers work with either imported bees of mainly *A. m. ligustica* and *A. m. carnica* origin, or with so-called "combination bees"; hybrids produced according the concepts of Brother Adam from Buckfast Abbey, Devon, UK. In spite of this very mixed origin, the discrimination of sub-species is not a task performed by local beekeepers. The main reason for this is the breeding structure amongst beekeepers. Denmark consists of more than 400 islands, so isolated mating areas are available for all sub-species kept by beekeepers. The regulation concerning mating areas is formalized into the Law of Beekeeping, and nearly 20 islands and a few peninsulas are formally recognised for the breeding of a single strain of bee. The mating areas offer high certainty for pure mated queens. It is not therefore considered necessary to employ additional tools to assure the pure status of the breeding material.

It is difficult to judge to what extent the *A. m. ligustica* and *A.m. carnica* populations in Denmark rely on importation of stock from their home range or other breeding populations. Isolated mating stations have been used for at least 20 years in Denmark. Even before the formal regulation was in place, Danish beekeepers were using isolated mating places and artificial insemination of queens. The "combination bee" population (often referred to as "Buckfast bees") rely on the import of foreign stock from most parts of the natural distribution of *A. mellifera*. The imported bees are, however, crossed into the local strain over three generations, and much of the variability brought in is lost again in that process.

For the past 20 years, the bees on the Island of Læsø have been investigated in great detail. The Læsø beekeepers have differing opinions about the best bees for local beekeeping. The commercial beekeepers on Læsø wanted freedom to choose the best bee for production of honey, whilst the majority of the beekeepers wanted to protect the local strain. Scientific investigations were carried out to determine the origin of the bees on Læsø and whether the bees were indeed a pure population of *A. m. mellifera*. The first investigations were based on measurements of the cubital index, a relative simple way to distinguish *A. m. mellifera* from the imported bees of *A. m. ligustica* and *A. m. carnica* origin (Svendsen *et al.*, 1992). Then followed two studies with molecular tools, first of mitochondrial DNA (Pedersen, 2002) and a detailed study of combining mtDNA and 11 microsatellites loci, comparing the bees of Læsø with a range of other populations of *A. m. mellifera* and a single population of Danish *A. m. ligustica* (Jensen *et al.*, 2005). Finally a project concerning the conservation of the Læsø bee was completed in 2008, using the 25 microsatellite loci, on 8,000 bees from Læsø collected between 2005 and 2007, compared with 3,000 bees from Austria, Croatia, Ghana, Italy, the former Yugoslav Republic of Macedonia, Norway, Poland, Slovenia, South Africa, Spain, Switzerland, and Turkey. Morphometrics performed at Oberursel, Germany by Stefan Fuchs on 195 of the bees from Læsø confirmed the status of part of the Læsø bees as *A. m. mellifera* bees (Moniteringsgruppen, 2008). The new conservation plan on the indigenous Læsø bee allows space for commercial beekeeping to coexist on the island. A follow up study of the population on Læsø is scheduled for 2010 or 2011 concerning the conservation goals, the effective population size related to long time sustainability, and the given border for the two populations on the Island to remain separated.

France

Situation for honey bee sub-species in France

Since the middle of the last century, French beekeepers have imported large numbers of queens of European sub-species other than the native *A. m. mellifera* to make hybrids which have been proved to be much more productive for honey and royal jelly production (Cornuet and Fresnaye, 1979). Whilst *A. m. mellifera* ecotypes had been described in different places in France in the sixties (Louveaux *et al.*, 1966), modern beekeeping including hive transportation and queen introductions has resulted in genetic mixing with local ecotypes. In some places, local honey bees have been completely replaced by hybrid strains. In other places, however, there is a real interest in the use and protection of the local ecotypes. Modern techniques have been developed in France using microsatellites, mitochondrial and morphological analysis to look at the genetic integrity of *A. m. mellifera* (Cornuet and Garnery, 1991; Garnery *et al.*, 1993; Estoup *et al.*, 1995). While scientists can use these methods to study specific honey bee populations (Strange *et al.*, 2008), the cost is still expensive for beekeeping applications. Recently, a new powerful morphological method that correlates morphometric and molecular data has been developed by the National Museum of Natural History in Paris to characterize honey bee colony genetic origins. This database is now available on internet to the beekeepers (http://apiclass.mnhn.fr).

Germany

In Germany, honey bee breeding has a long tradition and is well organized. There are about 140 registered mating stations, some of them well isolated on islands or in high mountain areas. In addition, about 100 insemination stations are in use to achieve controlled matings and to conserve different breeding populations. There are about 50,000 queens mated under controlled conditions per year, or more than 5% of the total number of colonies.

In addition to about 350 "*carnica*" breeders accredited by the German beekeeper association "Deutscher Imkerbund (DIB)", about 20 "Buckfast" breeders produce queens under the registration of the "Gemeinschaft der europäischen Buckfastimker (GDEB)". Apart from "*carnica*" and Buckfast, there are currently no other sub-species bred and propagated in Germany. While Buckfast breeders disregard any sub-species specific characters and base their selection exclusively on quantitative traits of economical interest, the DIB breeding guidelines demand conformity with sub-species specific characters for the registration of all "*carnica*" queen breeders. The relevant morphological characters have been described by Goetze (1964). Young offspring worker bees are checked for their pigmentation of tergites, hair length on tergite 5, width of tomentum on tergite 4 and the cubital index. Young offspring drones are checked for their pigmentation of tergites, the hair colour and the cubital index. Usually, 50 individuals of each sex are checked. According to the DIB guidelines, pure *A. m. carnica* bees have to meet the standards given in Table 4.

Studies based on more comprehensive morphometric characters and complex statistical analyses are used for the scientific discrimination of honey bee populations. At the bee institute in Oberursel, Ruttner established a large database with 38 standard characters of currently more than 3,000 samples of known origin. The data base serves as an international reference for the biogeography of honey bees (Ruttner, 1988).

Table 4. Morphometric standard for "*carnica*" bees according to DIB guidelines.

Character	Workers	Drones
Pigmentation of tergites	100% without colour spots or stripes, or max. 30% with spots	100% without colour spots or stripes, or max. 10% with spots
Hair length on tergite 5	100% < 0.35 mm, or max. 30% 0.35-0.40 mm	
Hair colour		100% grey or max. 20% brown
Width of tomentum	100% wider than stripe without hair, or max. 50% as wide as stripe without hair	
Cubital index	Average > 2,5, and less than 15% < 2.33	Average > 1.8 and 0 % < 1.4

Greece

Ruttner's (1988) morphometric analysis has led to the conclusion that the sub-species *A. m. adami*, *A. m. macedonica*, *A. m. cecropia* and *A. m. carnica* exist in Greece. Some of the Greek islands may still have pure populations. Despite the morphological differences among these sub-species and *A. m. cypria* from Cyprus, however, no significant differentiation has been observed based on mitochondrial and allozyme data (Bouga *et al.*, 2005a, b). In an attempt to promote the use of local honey bee sub-species and to identify the characteristics of each sub-species or strain produced commercially, a Certification System of Honey Bee Queens has been established at the Laboratory of Agricultural Zoology and Entomology of the Agricultural University of Athens, concerning the genetic origin, as well as the quality of the produced queens. During the first years of its establishment (2004-2007), the system was funded by the EU and the Hellenic Government (under Dir. 797/04 for beekeeping). The Laboratory of Agricultural Zoology and Entomology of the Agricultural University of Athens is the unique accredited Body in Greece, according to the criteria procedures of ELOT EN 45011.

Methodology used

PCR-RFLP is currently used to discriminate maternal lines of local versus imported honey bee populations. The procedure uses restriction enzymes which react with the mitochondrial gene segments of amplified 16s rDNA (*Ssp*I, *Dra*I, *Hinc*II, *EcoR*I, *Pst*I, *Alu*I) or COI (*Sau3A*I, *Fok*I, *Bcl*I, *Ssp*I, *Hinc*II), of local honey bees. In particular, for *A. m. macedonica* honey bees the diagnostic restriction enzymes, *Sty*I and *Nco*I, recognize one site on *COI* gene segment of mtDNA only in this sub-species. Research in progress will update the specifications (Martimianakis *et al.*, 2011).

Procedure followed

The queen breeder sends an application to the Laboratory at Athens each month for the queens they produce. This laboratory collaborates with that responsible for the quality of queens and they decide when samples will be collected. The sampling procedure is very strict and defined in advance in fine details by both accredited laboratories. Samples are collected in a rate of 10% of the examined queens, and samples are also taken from the mother colonies, for the determination of the queens' origin. All queens produced by these breeders must be marked with a specified colour given to them by the accredited bodies. All queen nuclei also need to be marked. After all analyses, the accredited body produces a Certificate, which is unique for every month and is not allowed to be reproduced by the bee breeder. The establishment of the certification system took almost two full years and the system was put in practice during the third year (2007). Since then certificates have been produced for four bee breeders every year.

Italy

Italy hosts two honey bee sub-species: *A. m. ligustica* Spinola in continental Italy, and *A. m. sicilana* Grassi in Sicily. According to morphometric analyses (Ruttner, 1988) both of them were included within the evolutionary branch C. Beyond detecting a hybrid origin for both Italian sub-species (Franck *et. al.*, 2000; Marino *et al.*, 2002), molecular analyses has confirmed branch-C membership for *A .m. ligustica*, while *A. m. siciliana* has been moved into the African evolutionary A-lineage (Sinacori *et al.*, 1998).

Because of its large honey storing ability, docility, low swarming propensity and adaptability to a wide range of climatic conditions, *A. m. ligustica* queens have been exported worldwide for more than 150 years (Bar-Cohen *et al.,* 1978; Sheppard, 1989; Woodward, 1993). In 2002, 5% of the queens produced were exported to Northern Europe and 1% to the rest of Europe, Australia, New Zealand and the USA. 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). Given the success and scale of the queen breeding industry, the conservation of Italian honey bees is of major concern for economic reasons alone, apart from the biodiversity perspective,

where a priority is laid on preserving the endemic sub-species of honey bees in Europe.

Apis mellifera 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* in the northwest and with *A. m. carnica* in the north eastern Friuli region (Comparini and Biasiolo, 1991; Nazzi, 1992; Meixner *et al.*, 1993) These two sub-species also belong to the 'C' lineage.

The Sicilian bee population differs from the mainland one through specific adaptations to some of the region's flora (Sinacori *et al.*, 1998), to a subtropical Mediterranean climate with hot, dry summers and to peculiar enemies (Ruttner, 1988). *A. m. siciliana* characteristics include production of many swarm cells, swarming delayed until after virgins have hatched, and no flight during the hottest hours of the day (Ruttner, 1988; Amodeo, 2003). Although Sicilian beekeepers have occasionally imported *A. m. ligustica* since the beginning of the 20th century, bees from northern Italy have been introduced on a large scale only recently and predominantly in the eastern part of the island. They are now spreading into other parts and hybridising with the local bee (Badino *et al.*, 1985). Nevertheless, the "black bees", the nickname used by Sicilian beekeepers for their native sub-species, are preferred by beekeepers in the droughty central area of the island because of their capability to reduce or interrupt the brood rearing during the summer when neither pollen nor nectar are available, which acts as a natural control of reproduction of the parasitic mite *Varroa destructor*.

Genetic variability of the Sicilian sub-species has been studied in detail using allozymes (Badino *et al.,* 1985; Biondo *et al.*, 1991); in particular the S allele at the EST locus has been defined as a marker for the bees (Badino *et al.*, 1985). The mitochondrial genome of Sicilian bees has been studied in the tRNAleu-cox2 intergenic region mainly with RFLPs: local populations investigated by *Dra*-I test showed high frequencies (up to 100% in some of them) of A-branch mitotypes (Garnery *et al.*, 1993; Sinacori *et al.*, 1998, Franck *et al.*, 2000). Further investigations using microsatellites indicated that the nuclear genome has been introgressed with genes from south-eastern European sub-species and there has been probable hybridization with *A. m. mellifera* (Franck, 2004).

In late 1980s some *A. m. siciliana* colonies were investigated using morphometric analysis and allozyme variability, and the results were compared with standard reference material. The most distinctive samples were isolated on the island of Ustica for conservation purposes; ten years later three different breeding lines were moved from Ustica to different islands of the Sicilian archipelago "Eolie" (Filicudi, Alicudi and Vulcano), where since 2004 an official conservation programme is ongoing. These three populations serve as a source of genetic material for outcrossing to selected stock.

In 1997 the national register for professional queen breeders was founded by the Ministry of Agriculture (Mipaaf, 1997) with the objective of safeguarding and improving the native sub-species. Two different subsections (one each for *A. m. ligustica* and *A. m. siciliana* breeders) certify the production of breeders able to provide queens that correspond to specific standards, thereby offering beekeepers the possibility of buying queens that meet biological, genetic and health requirements. It is estimated that 120.000 queens are produced annually in Italy, half of these by registered breeders.

Registered breeders yearly submit to CRA-API (the former Italian National Institute for Beekeeping) samples for morphometric analyses, which is the official analytical method as described in the national register regulation. Samples are constituted of 40-50 adult worker bees per lineage to be certified; bees are collected and immediately stored in absolute ethanol. A set of 20 right forewings per sample are pasted on a photographic slide and then scanned with a 3,200 dpi resolution. Total wing length (F_L) and width (F_B) measures are taken with a stereomicroscope provided of an ocular micrometer, while 19 points (Fig. 3) are manually acquired and stored in the Databees software (Ep-Informatica, 2003), allowing the measurement of the following 30 characters (Du Praw, 1964; Bruckner, 1976; Ruttner *et al.*, 1978) for analyses (Fig. 4): 17 angles (A1, A4, B3, B4, D7, E9, G7, G18, H12, J10, J16, K19, L13, M17, N23, O26, Q21), 7 linear measures (Radial field, Inner wing width, Inner wing length, A, B, C, D), 5 index (Discoidal shift, Cubital, Precubital, Dumb-bell, Radial) and one area (Area 6).

For each sample, the mean and the standard deviation of each character are calculated and the data are compared with a reference database that includes sub-species of Italy and neighbouring countries. The probability of membership in each reference population is then given by a multivariate analysis with *Statistica* software (ver.7, StatSoft Italia s.r.l.). Single sample assignment is completed if percentage of confidence to any of the reference sub-species is >95%, otherwise morphometric analyses continue with the addition of new characters, including colour and width of abdominal tergites. For samples not assigned to any of the reference populations, an analysis of variance is then performed to evaluate amongst the individual characters which are the ones most affecting the result.

Molecular tools can be used in parallel to the official morphometric method to confirm results or to unveil details concerning samples that are not unambiguously assigned to any of the reference population. Both mitochondrial and nuclear markers can be used; the first (*Dra*-I test on a single honey bee worker, Garnery *et al.*, 1993) is preferably used when sample is under suspicion of hybridization among taxa from different evolutionary lineages (i.e., *A. m .ligustica* and *A. m. mellifera*, or *A. m. siciliana* and *A. m. ligustica*), while the analysis of nuclear data (set of 8 microsatellite loci A113, A28, A(B)24, A14, A107, A88, Ap43, A7 from Estoup *et al.*, 1995) is an additional tool used to detect hybridization among taxa within the same evolutionary branch (i.e. *A. m. ligustica* and *A. m. carnica*). Mitotypes scored with

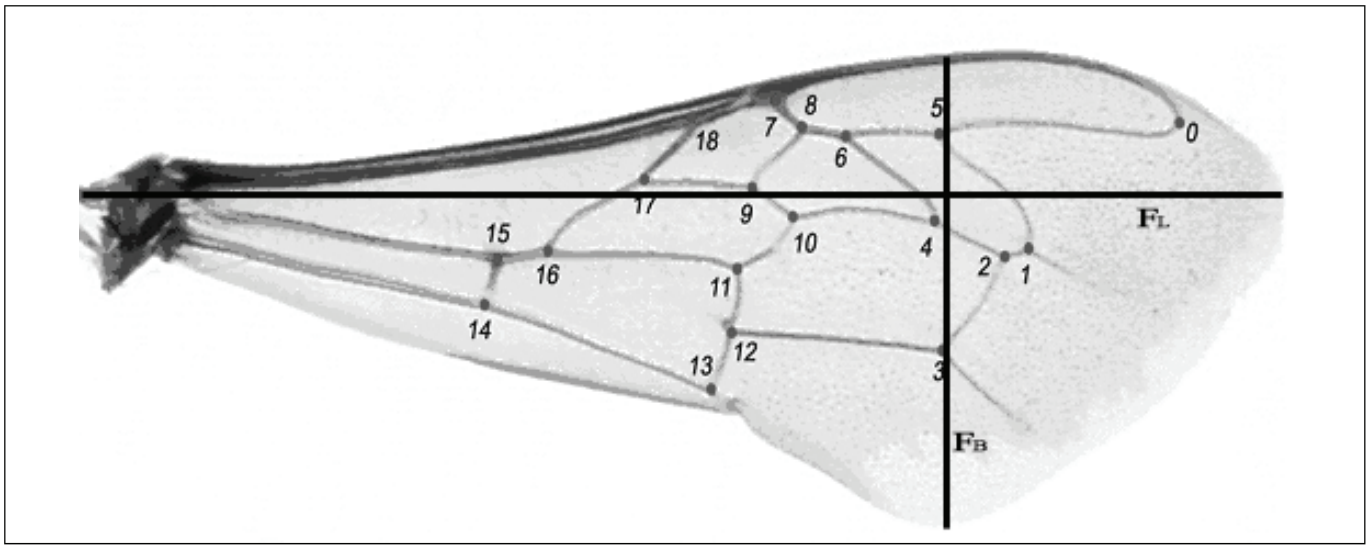

Fig. 3. Morphometric analyses used in Italy: Wing points (0 to 18) are manually acquired, whilst wing length (F_L) and width (F_B) are measured by stereomicroscope.

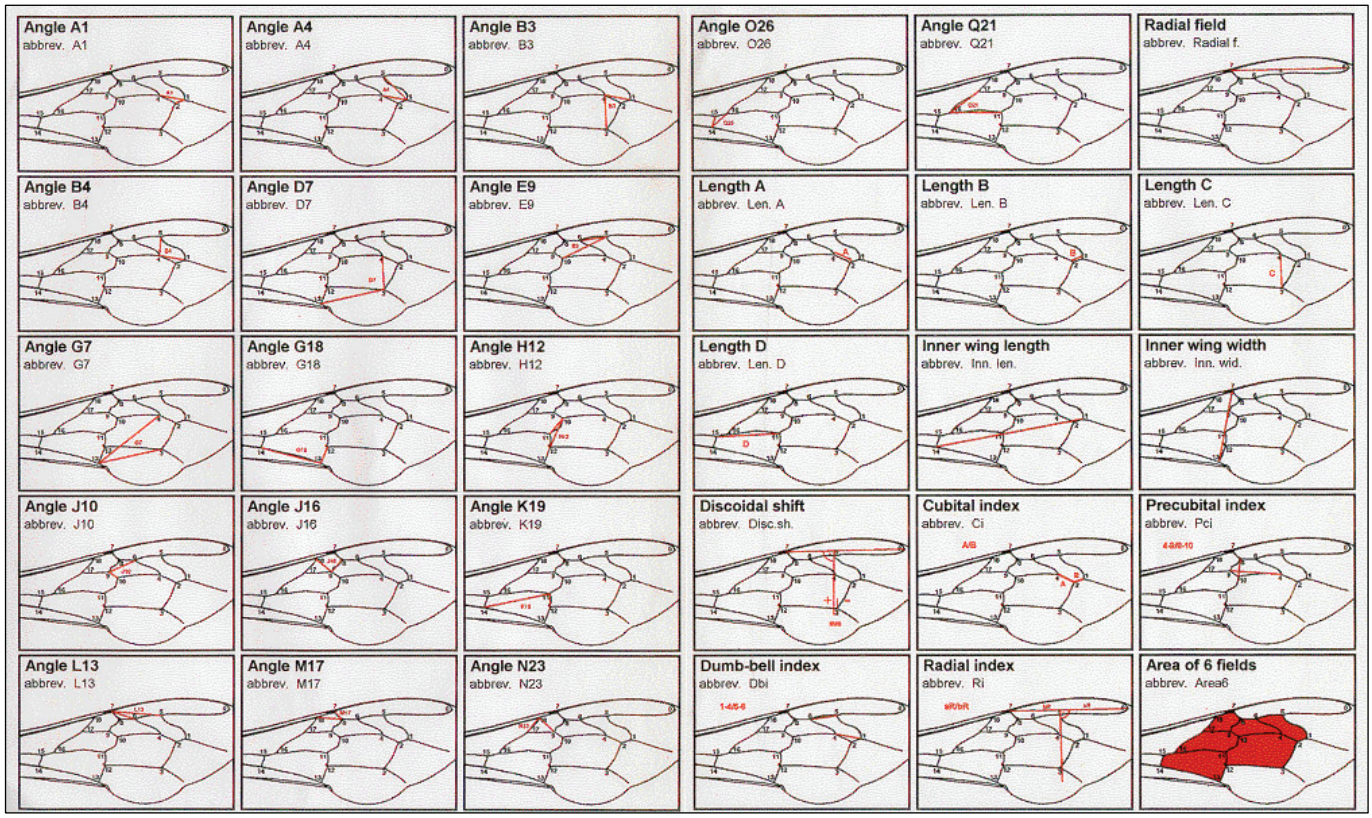

Fig. 4. Morphometric analyses used in Italy. Figure kindly provided by Bee Research Institute, Dol, Czech Republic.

mitochondrial markers are scored on agarose gel and confirmed by direct sequencing only in case of new haplotype detection; individual microsatellite data are added to a reference database that has been created by CRA-INA with funds of BABE European project that includes over 300 records. Individual assignment is performed with the software GeneAlex (Peakall and Smouse, 2006) and corroborated with Structure ver. 2.2 (Pritchard *et al.*, 2000); due to the unbalanced database composition (most of the records from C-branch samples) the proportion of membership threshold has been set at 95% for A-branch taxa, while it has been lowered at 80% for C-branch taxa.

Despite detecting a high level of genetic variability, the most recent study on large scale on *A. m. ligustica* (Dall'Olio *et al.,* 2007) highlighted the absence of genetic structure within and among the sampled *A. m. ligustica* subpopulations, confirming that the Italian honey bee population has become a unique large one, probably as a result of intensive beekeeping practices such as migratory beekeeping

and large-scale commercial queen trading. Preliminary data from a recent survey of *A. m. siciliana* (Dall'Olio *et al.,* 2008) underline the efficacy of the conservation programme and demonstrate the existence of areas in the western side of Sicily where colonies have traits consistent with those on the conservation island.

Norway

Archaeological finds of the dark European honey bee *A. m. mellifera* in Norway have been dated back to the year 1100, but beekeeping known from written sources goes back only 250 years (Ruttner *et al.*, 1990). During the 20th century several sub-species such as *A. m. ligustica*, *A. m. caucasica*, and *A. m. carnica*, and the hybrid Buckfast, were imported to Norway, which is the northern border for the natural distribution of honey bees. Nowadays the 3,000 beekeepers in Norway, having all together 60,000 colonies, can choose between three major breeds or sub-species after *A. m. ligustica* and *A. m. caucasica* were found to be inferior compared to the other sub-species under the prevailing climatic conditions. Based on a questionnaire in 2008 among 20% of the beekeepers in Norway, *A. m. carnica* is the most common sub-species (46% of the 60,000 colonies in Norway), followed by *A. m. mellifera* (29%), Buckfast (13%); and as a result of the different sub-species there are quite a few colonies of mixed sub-species (12%). The previously widespread *A. m. mellifera* is now rare in Europe and Norway has among the largest remnant populations of this sub-species. The Norwegian Beekeepers Association is responsible for national breeding programmes for *A. m. carnica* and *A. m. mellifera* bees where mating takes place at isolated mating stations to avoid hybridization. To avoid hybridization in the standing populations of the different sub-species there is an area about 3500 km^2 where beekeepers are allowed to keep *A. m. mellifera* only. In several other areas *A. m. carnica* is the only sub-species to be kept. These official areas with only one sub-species of honey bees are regulated by law. In addition, beekeepers of a district often agree on one sub-species to avoid hybrids which often are aggressive in the second and third generation. So far morphometric traits, especially the cubital index, have been used as a simple method to remove hybrids of *A. m. mellifera* and *A. m. carnica*. In later years the national breeding programme and some breeders have used the DAWINO method (www.beedol.cz) which includes coordinates of 19 intersections of forewing veins and which computes the probability by which the bee sample belongs to each of the sub-species. In connection with the effort to conserve the *A. m. mellifera* at Læsø, Denmark, samples of Norwegian *A. m. mellifera* have been analysed with molecular techniques using 25 microsatellite loci. The result confirmed the status of the Norwegian *A. m. mellifera*. In the future molecular methods will play a more important part to distinguish the different sub-species of honey bees in Norway.

The cubital index on the forewing is measured for honey bee colonies scheduled for breeding of queens and drones. The procedure is according the recommendation by Ruttner (1983). The worker bees of queens in the national honey bee breeding programme also undergo this measurement which is a simple method to distinguish *A. m. mellifera* from *A. m. carnica*. Buckfast bees, however, often have an intermediate cubital index which makes this method less suited in some cases. In recent years, more sophisticated morphological methods have been used to make sure that the breeding populations in the national breeding programme are pure. This includes the software programme CBeeWing and the Dawino analysis which measures more coordinates on the honey bee forewing. Whereas the CBeeWing gives the percentage of bees that are within the morphological limits of the sub-species in question, the Dawino analysis (which uses more wing measurements than the CBeeWing) gives a statistical probability that the samples are taken from the respective sub-species. Surprisingly, samples of bees from the same colony can be judged quite different in the two analyses. Due to the low density of honey bees in Norway, mating stations can be located where no honey bee colonies are found within 10-20 km radii. Some mating stations are regularly tested for the absence of alien drones by placing virgin queens at the mating station before the drone producing colonies are brought to the mating station.

In June 2007 and 2008, samples of worker bees were collected from bee colonies of *A. m. carnica* (sampled in June 2007 and May 2008) and *A. m. mellifera* (sampled in June 2008) included in the performance testing in the national honey bee breeding programme. The beekeepers who collected the samples were told to sample young bees to avoid sampling those that might come from neighbouring colonies. In 2007 the cubital index and the discoidal shift angle on the right forewing were calculated using the CBeeWing software. In May 2008 worker bees from the same colonies that were sampled in 2007 were collected again after emerging under a cage on the brood frame. A few days after hatching the right forewing of the young worker bees from the cages were analysed with the Dawino method. The June samples of *A. m. mellifera* (young bees, but not from under a cage) were analyzed with the Dawino method. The CBeeWing and Dawino analyses were based on measurements of 30 and 16-18 worker bees, respectively.

The measurements of the cubital index and the discoidal shift angle in the two samples taken from each *A .m. carnica* queen and analyzed by the two different methods were correlated (cubital index**:** $F = 14.45$, $P < 0.001$, $R^2 = 0.38$; Discoidal angle: $F = 84.31$, $P < 0.0001$, $R^2 = 0.78$). Using the measurement of the cubital index and the discoidal shift angle obtained in the Dawino analysis and the criteria set for *A. m. carnica* in the CBeeWing, however, gave quite different results between the two samples from the same queens (Table 5).

Table 5. Results of racial classification of *A. m. carnica* colonies using two different methods for sub-species classification. The Dawino method gives the probability of the sample to belong to the sub-species, whilst the CBeeWing method gives the percentage of wings within the range for *A. m. carnica*. CI and DSA denote the cubital index and discoidal shift angle respectively.

Colony	Dawino	CBeeWing	CI Dawino	CI CBeeWing	DSA Dawino	DSA CBeeWing
1	83% *A. m. carnica*	90%	2.82	2.85	6.40	4.70
2	85% *A. m. carnica*	100%	3.00	2.70	5.20	4.10
3	78% *A. m. carnica*	83%	2.87	3.08	4.10	2.00
4	87% *A. m. carnica*	90%	3.27	2.87	6.10	4.10
5	79% *A. m. carnica*	100%	2.76	3.14	6.40	5.50
6	81% *A. m. carnica*	100%	3.20	3.08	6.60	4.70
7	82% *A. m. carnica*	90%	2.86	2.79	5.10	2.60
8	85% *A. m. carnica*	100%	2.89	2.94	6.70	4.60
9	74% *A. m. carnica*	93%	2.65	2.85	6.00	5.40
10	87% *A. m. carnica*	100%	3.15	2.92	5.70	3.70
11	86% *A. m. carnica*	93%	2.82	2.96	4.40	3.10
12	91% *A. m. carnica*	100%	3.28	3.34	6.30	5.10
13	65% *A. m. carnica*	94%	2.71	2.69	6.30	4.60
14	72% *A. m. carnica*	93%	2.90	2.74	6.80	4.70
15	86% *A. m. carnica*	97%	3.39	3.21	5.60	4.30
16	63% *A. m. carnica*	100%	3.06	2.85	7.20	5.00
17	89% *A. m. carnica*	73%	3.07	3.15	3.80	1.70
18	84% *A. m. carnica*	97%	3.19	2.99	7.60	5.70
19	85% *A. m. carnica*	97%	2.88	3.03	6.20	3.00
20	78% *A. m. carnica*	100%	3.28	3.21	7.90	5.70
21	85% *A. m. carnica*	90%	3.06	2.87	3.70	2.20
22	77% *A. m. carnica*	93%	2.79	2.76	6.50	4.40
23	77% *A. m. carnica*	93%	3.26	3.07	5.90	2.80
24	85% *A. m. carnica*	83%	3.11	2.91	4.10	2.50
25	86% *A. m. carnica*	83%	3.35	2.93	5.70	2.90
26	82% Buckfast	76%	2.37	2.55	2.00	1.10

The proportion of wings within the range defined for *A. m. carnica* in the CBeeWing was not significantly related to the probability that the sample came from *A. m. carnica* colony ($F = 0.85$, $P = 0.366$, $R^2 = 0.04$). The Dawino analysis of the *A. m. mellifera* in 2008 suggested that some queens should not be used as breeder individuals because they were not pure *A. m. mellifera*. Using the criteria for pure *A. m. mellifera* used by CBeeWing and Nordbi, the Swedish organization for *A .m. mellifera*, gave quite different results, mainly because pure *A .m. mellifera* according to their criteria should have a negative discoidal shift angle. The probability of the sample belonging to *A. m. mellifera* was more strongly related to the average cubital index of the sample ($F = 70.82$, $P < 0.001$, $R^2 = 0.69$) than to the average discoidal shift angle ($F = 14.46$, $P < 0.001$, $R^2 = 0.31$). The percentage of wings within the limits for *A. m. mellifera* in CBeeWing conversely was more strongly related to the average discoidal shift angle of the sample ($F = 74.77$, $P < 0.001$, $R^2 = 0.70$) than to the average cubital index of the sample ($F = 19.50$, $P < 0.001$, $R^2 = 0.38$). The results from the Dawino- and CBeeWing analysis were, however, correlated ($F = 36.70$, $P < 0.001$, $R^2 = 0.534$) (Table 6).

To be able to sort hybrids from pure sub-species, it is of critical

Table 6. Results of racial classification of *A .m. mellifera* colonies using two different methods for sub-species classification. The Dawino method gives the probability of the sample to belong to the sub-species, and the CBeeWing method gives the percentage of wings within the range for *A. m. mellifera*. CI and DSA denote the cubital index and discoidal shift angle respectively.

Colony	Dawino	CBeeWing	CI (Dawino)	DSA (Dawino)
1	50% *A. m. carnica*	10%	2.41	2.5
2	64% *A. m. mellifera*	20%	1.79	1.0
3	67% *A. m. mellifera*	10%	1.99	1.1
4	97% *A. m. mellifera*	60%	1.60	-1.0
5	97% *A. m. mellifera*	70%	1.74	-2.0
6	44% *A. m. mellifera*	11%	2.03	1.5
7	97% *A. m. mellifera*	70%	1.60	-1.2
8	84% *A. m. mellifera*	50%	1.77	-1.7
9	68% *A. m .ligustica*	11%	2.23	3.4
10	99% *A. m. mellifera*	70%	1.62	-0.7
11	81% *A. m. mellifera*	50%	1.81	-0.6
12	96% *A. m. mellifera*	40%	1.71	0.1
13	100% *A. m. mellifera*	40%	1.65	2.4
14	89% *A. m. mellifera*	30%	1.96	-0.9
15	93% *A. m. mellifera*	35%	1.62	-0.1
16	78% *A. m. mellifera*	35%	1.88	1.3
17	92% *A. m. mellifera*	56%	1.85	0.1
18	87% *A. m. mellifera*	68%	1.77	-1.0
19	65% *A. m. mellifera*	10%	1.87	1.3
20	85% *A. m. mellifera*	28%	1.73	1.0
21	76% *A. m. mellifera*	25%	1.82	0.7
22	99% *A. m. mellifera*	65%	1.44	-0.9
23	95% *A. m. mellifera*	53%	1.70	-0.3
24	97% *A. m. mellifera*	72%	1.67	-2.1
25	88% *A. m. mellifera*	70%	1.81	-1.1
26	92% *A. m. mellifera*	25%	1.72	1.2
27	47% *A. m. carnica*	0%	2.25	3.8
28	77% *A. m. mellifera*	45%	1.93	0.2
29	48% *A. m. carnica*	5%	2.33	2.8
30	88% *A. m. mellifera*	80%	1.75	-3.0
31	87% *A. m. mellifera*	55%	1.84	-0.9
32	88% *A. m. mellifera*	60%	1.7	-0.7
34	95% *A. m. mellifera*	40%	1.72	0.7
35	96% *A. m. mellifera*	40%	1.65	-0.2
36	93% *A. m. mellifera*	40%	1.66	0.0
37	85% *A. m. mellifera*	40%	1.79	-0.5
38	57% *A. m. mellifera*	5%	2.12	1.5

importance that the bees sampled from a colony actually belong to that colony and do not come from a neighbouring one. The variation in the cubital index and discoidal shift angle between the two samples suggest that the beekeepers that collected the first sample did not solely sample young bees from the colony, but also included bees from neighbouring colonies. The beekeepers must therefore be trained how to sample to be sure that the sample only consists of young bees from a single colony. Another source of variation is measurement error, but the repeatability of wing measurements such as the cubital index is reported to be high (SD = 0.1 (http:// www.cybis.se/cbeewing/index.htm).

In our opinion CBeeWing is not suitable for detection of hybridization in *A. m. carnica* populations, and we question the criterion of a negative discoidal shift angle for pure *A. m. mellifera*. This criterion has been used in Sweden for many years, but has never been used in Norway, and the discoidal shift angle does not seem to influence the results obtained in the Dawino analysis as strongly as the cubital index. For *A. m. mellifera* it seems that sufficient information on hybridization can be extracted from measurement of the cubital index.

Poland

Honey bee breeding in Poland has been regulated by governmental law for over 30 years for three honey bee sub-species: *A. m. mellifera* (commonly called the "national", "local" or "black" bee); *A. m. carnica*: and *A. m. caucasica*. The territory of Poland is a natural habitat of *A. m. mellifera*. Polish beekeepers started to import Caucasian queens from countries of the former USSR and Carniolan queens, mainly from Danubian countries and Austria, during the last century. Uncontrolled importation resulted on hybridization of bee stocks. The imported sub-species of bees, especially the Carniolan bees, began to dominate. Closed honey bee breeding regions were created in order to protect national sub-species (*A. m. mellifera)*. Four ecotypes are protected, the Augustowska, Północna, Kampinoska and Asta lines. The purpose of keeping these natural populations is to protect and conserve the natural genotype. At present, about 800 colonies of Augustowska bees, 900 colonies of Kampinoska bees and 360 colonies of Asta bees are kept in these isolated zones.

Two types of breeding programmes exist in Poland. One is for genetic improvement of the sub-species and the other is a hybrid programme in which the breeders are trying to get very productive hybrids from two sub-species or from different breeding lines within a sub-species. Over 90% of breeding apiaries in Poland use instrumental insemination to produce queens for productive colonies (Bieńkowska *et al*., 2008; Woyke *et al*., 2008). In the 20th century *A. m. caucasica* bees were imported from the mountains of Caucasus. The bees have the smallest body size and the longest proboscis (6.70 to 7.25 mm) of European bees. There currently are eight Caucasian breeding lines being reared in Poland.

Apis mellifera carnica occurs naturally in southern Poland. There are a few local breeding lines (e.g. Pogórska, Beskidka and Dobra) that have been conserved and improved in their native regions. In addition, there are 34 lines of *A. m. carnica* bees that were imported from Austria, Germany, Hungary and the former Yugoslavia (Bieńkowska *et al*., 2008), which have been bred for improved performance. The lines are bred in different regions of Poland with a view to weather and flow conditions.

Measurements of the width of tergite IV, the length of the proboscis and the cubital index are used to define the sub-species in Poland. The measurements are made on 30 bees per colony, and the arithmetical mean value of each trait is calculated. The average values of the traits are compared to standard values known for each sub-species (Table 7) (Bornus and Gromisz, 1969; Gromisz, 1981). If the calculated means diverge by more than -3 to + 3 from the standard, the conclusion is that the colony does not belong to the specified sub-species. The construction of models were based on morphological investigations of several bee lines: *A. m. mellifera* (Bornus *et al*., 1966; Gromisz and Bornus, 1971); *A. m. carnica* as it occurs in Poland (Bornus and Gromisz, 1969); and *A. m. caucasica* found in the former USSR (Gromisz, 1978).

Table 7. The average level of value of traits in model populations in Poland.

Sub-species of bees	**Width of IV tergite (mm)**	**Length of proboscis (mm)**	**Cubital index (%)**
	Average	Average	Average
A. m. mellifera	2.356	6.115	61.4
A.m. carnica	2.302	6.458	51.2
A.m. caucasica	2.242	6.976	54.7

The length of proboscis and the cubital index have been estimated since 2008. Based on the results obtained during 2007 and 2008, it has been estimated that about 99% of colonies of *A. m. carnica* belong to this sub-species. From year to year, the percentage of *A. m. caucasica* colonies which meet the standard for the length of the proboscis has diminished while more colonies meet the standard for the cubital index. The apiaries which are in closed regions of breeding have had difficulties meeting morphological standards; in 2007 and 2008, only 80% of the colonies of *A. m. mellifera* met the standards for the cubital index (Rostecki *et al.,* 2007) (Table 8).

Using the cubital index to determine sub-species of bees (KauhausenKeller, 1991) is not always effective because the ranges of its values overlap (Ruttner, 1988). There has been an effort in Poland to use wing venation characteristics as an alternative for discriminating sub-species. A method to automatically measure wings using image

Table 8. Number and percentage of Polish bee samples classified to sub-species according to morphological traits in 2007 and 2008.

Sub-species	Breeding line	2007							2008				
		No of colonies	Proboscis		IV tergit		Cubital		No of colonies	Proboscis		Cubital	
			n	%	N	%	n	%		n	%	n	%
A. m. mellifera	Asta	35	35	100	35	100	35	100	44	44	100	40	90.9
	Kamp.	39	32	82.0	39	100	34	87.2	33	21	63.6	31	93.9
	August.	26	24	92.3	24	92.3	18	69.2	30	27	90.0	26	86.6
	Półn.	42	36	85.7	39	92.8	26	61.9	73	55	75.3	47	64.3
	total	142	127	89.4	137	96.5	114	80.3	180	97	53.8	144	80.0
A. m. carnica	CT46	24	23	95.8	24	100	24	100	10	9	90.0	10	100
	Pogórs.	21	21	100	21	100	21	100	20	20	100	20	100
	Dobra	43	43	100	43	100	43	100	51	51	100	51	100
	Beskid.	-	-	-	-	-	-	-	37	37	100	37	100
	Remaining lines (n = 34)	673	668	99.2	673	100	672	99.8	574	571	99,5	573	99.8
	total	761	755	99.2	761	100	760	99.8	692	688	99.4	691	99.8
A. m. caucasica	(8 lines)	108	75	69.4	108	100	97	89.8	79	47	59.5	78	98.7
Total		1011	957	94.6	1006	99.5	971	96.0	951	932	87.5	913	96.0

analysis has been developed (Tofilski, 2004, 2005; Gerula *et al.,* 2009a,b). Co-ordinates x and y of 19 wing vein intersections were measured and analysed as 38 variables. Classification is based on 29 variables chosen from standards for each sub-species. On this basis, 94.4% of investigated colonies were distinguished correctly. The highest probability of discrimination was for *A. m. carnica* (97.5%) and the lowest was for *A. m. caucasica* (71.4%). Canonical analysis showed that *A. m. mellifera* colonies differed from the other sub-species, but those measurements for *A. m. carnica* and *A. m. caucasica* colonies overlap. Development of the method continues (Gerula *et al.,* 2009a,b).

Portugal

Honey bee sub-species in Portugal

No systematic study has been done in Portugal to demonstrate which sub-species of *A. mellifera* are currently the most widespread. There is no reason to doubt, however, that the most represented sub-species is *A. m iberiensis.* This bee is morphometrically and genetically placed between the northern African *A. m. intermissa* and the north-western European *A. m. mellifera*. Morphometric and molecular genetic studies quantitatively ascribing sub-species dominance in beekeeping are lacking, despite the fact that most professional beekeepers appear pleased to think they are indeed using the bee stock "native" to the country. Although samples of honey bees from Portugal have been studied abroad for sub-species allocation, their representativeness is clearly insufficient to provide an overview on this issue.

Hence the "temptation to test foreign sub-species" mainly results from some beekeepers' belief that local honey bees are inherently unable to deliver the high annual honey production needed to compete in a global market, no matter how well they thrive in the environmental conditions of Portugal. Unlike *A. m. ligustica* and *A. m. carnica*, the honey production potential of *A. m. iberiensis* has not yet been subjected to a scientifically based genetic improvement programme, although efforts to genetically enhance the local honey bee strain have just started. This work would benefit from a national system for sub-species certification. A few Portuguese Universities have used morphometrics to assign sub-species identity to geographic populations. The University of Evora plays a major role in this field, particularly in discriminating *A. m. iberiensis* from *A. m. ligustica* and *A. m carnica*. Most of these sub-species authentications have, however, been done within larger research projects (on areas of apidology other than systematics), meaning that this kind of work chronically tends to remain hidden within these projects.

Methods for sub-species allocation

Thirty workers are sampled from within each colony and preserved in 95% ethanol at 5ºC. Fifteen workers per colony are dissected and their proboscis, right side wings, right hind leg and 4th abdominal tergite are mounted on 35-mm slides. Images are projected on a digitising table for measuring using SigmaScan software. Previous discriminant statistical analyses have allowed building a set of morphometric variables with the highest discriminant power between *A. m. iberica*, *A. m. ligustica* and *A. m. carnica*. Thus, out of an initial set of 30 studied variables, presently we use only 14 variables (Table 9) in factorial analysis for discriminating between these 3 sub-species. Standard values for these variables for the three sub-species of interest were provided by the Institut für Bienenkunde (IFB; Oberusel, Germany). The measurements obtained from colonies of interest are compared to subspecific reference values provided by the IFB by factorial analysis (PCA factor extraction). Frequently the first three extracted factors are able to explain 70 to 80% of the variability of the data. Scores obtained for each colony in tri-dimensional factorial space are then plotted together (and with the subspecific reference scores), providing for a visual and statistical interpretation of the probable subspecific placement of each colony. Table 9 provides a summary of the data collected from Portuguese colonies that we have considered as belonging to *A. m. iberiensis*, *A. m. carnica* or *A. m. ligustica*.

Former Yugoslav Republic of Macedonia (UN temporary reference)

Apis mellifera macedonica (in the north) and *A. m. carnica* (in the south) appear to introgress in the territory of the former Yugoslav Republic of Macedonia. Early observations (Alpatov, 1948a; Brother

Table 9. Summary of morphometric variables measured on honey bees from Portuguese colonies.

Variables *(Mean ± standard error of mean)*	**Sub-species**		
	A. m. iberiensis	***A. m. carnica***	***A. m. ligustica***
Lengths (mm):	-	-	-
proboscis	6.545 ± 0.054	6.257 ± 0.081	6.389 ± 0.083
femur	2.705 ± 0.007	2.615 ± 0.008	2.662 ± 0.009
tibia	3.116 ± 0.012	2.983 ± 0.015	3.009 ±0.009
metatarsus	2.190 ± 0.011	2.103 ± 0.009	2.113 ± 0.013
forewing	9.086 ± 0.029	9.235 ± 0.026	9.303 ± 0.027
Widths (mm):	-	-	-
forewing	3.014 ± 0.012	3.153 ± 0.015	3.159 ± 0.011
hindwing	1.714 ± 0.016	1.833 ± 0.007	1.889 ± 0.010
Forewing angles (degree):	-	-	-
G18	98.520 ± 0.637	96.863 ± 0.367	94.490 ± 0.457
K19	82.460 ± 0.459	79.157 ± 0.691	81.641 ± 0.501
L13	14.450 ± 0.224	12.608 ± 0.446	12.818 ± 0.261
J16	104.489 ± 0.453	100.753 ± 0.593	99.289 ± 0.589
O26	44.774 ± 0.532	39.958 ± 0.567	39.639 ± 0.609
Cubital index (mm):	-	-	-
numerator	0.473 ± 0.006	0.548 ± 0.011	0.529 ± 0.007
Pigmentation: [from 1 (black) to 5 (orange)]:	-	-	-
4th tergite	1.313 ± 0.038	2.148 ± 0.075	3.675 ± .081

Adam, 1968; Ifantidis, 1979; Ruttner, 1988) indicated that these two honey bee sub-species are present and the morphometric results (Sljahov, 1973, 1979; Naumovski and Krlevska, 1996; Naumovski *et al.*, 2000; Kiprijanovska and Uzunov, 2002; Uzunov, 2007; Uzunov *et al.,* 2009) support the existence of two sub-species and undefined genotypes. The native honey bees are highly adapted to the strong seasonal variation during the year. There is rapid spring development followed by rapid reduction of the brood area during the mid summer. Some years there is manifestation of a high swarming tendency which ends together with summer brood reduction. The bees typically are gentle. Short winters occasionally result in no broodless period during the whole year.

The populations of honey bee in the country were mainly discriminated by using three major methodologies, such as:-

Morphometrics

First (Ruttner, 1988) wing morphological examination were primarily focused on length of proboscis and fore wing, Cubital Index, Ven. angles E9, G18, J10, L13 etc. Recently (Uzunov *et al.*, 2009), 1,800 samples from the whole country were analyzed for 21 wing characteristics with classical morphometrics and by using Beewings 1.20 software (DAWINO method, www.beedol.cz). The following wing characteristics were used: angles A1,A4, B3, B4, D7, E9, G7, G18, H12, J10, J16, K19, L13, M17, O26, G21, indexes CI, PCI, DBI, RI and AREA 6. The data were statistically analysed and compared with the data from reference samples from five sub-species, such as: *A. m. carnica*, *A. m. macedonica*, *A. m. ligustica*, *A .m. mellifera* and *A .m. caucasica*. The average relationship of the analyzed honey bees from the selected apiaries to the other five sub-species is found to be: *A. m. macedonica* (15.87%); then *A. m. ligustica* (12.70%); *A. m. carnica* (10.32%) and at the end *A. m. mellifera* (4.76%); and *A. m. caucasica* (4.76%). The same methodology is now implemented as a regular routine for first level of honey bee sub-species determination in the country.

Molecular methods

Fifty samples of colonies recently were collected from 22 different locations across the country. Samples are being analyzed for variation at 25 microsatellite loci. These bees will be compared to neighbouring populations from Albania, Bulgaria, Greece and Serbia. The research is ongoing.

Performance testing

Based on the recent morphometrical and molecular discrimination of the populations of honey bees in the country, a long term performance testing protocol is implemented by the honey bee laboratory under the framework of Faculty for Agricultural Sciences and Food from Skopje. The testing of the populations is focused on evaluation of total of 28 morpho-anatomical, physiological, biological, ethological and productivity characteristics. The initial testing is organized in five apiaries in three different regions of the country.

The performance testing protocol is implemented by regular measurements on three weeks during the active beekeeping breeding season (March – November). Seasonal dynamic and variations of the number of open and sealed brood cells, number of bees, swarming behaviour, quantity of pollen and honey among different queen sister groups (*A. m. macedonica*) is in main focus of the protocol. Additionally, other characteristics such as: defensive behaviour, hygienic behaviour and disease status (infestation level of *V. destructor*, virus incidence, *Nosema* incidence and *Nosema* species determination) are tested for comparative analysis with the other characteristics mentioned. The whole activity is in cooperation with the Kirchhain bee institute, Germany, the Agricultural Institute, Ljubljana, Slovenia and the Faculty of Agricultural Sciences, Aarhus, Denmark.

Romania

Romania is within the natural area of distribution of *A. m. carnica*. A prior study (Foti, 1965), using morphometric and biological criteria, showed some differences between Romanian honey bees and other *A. m. carnica* populations, resulting in the proposed standalone sub-species *A. m. carpathica*. This sub-species was not confirmed by Ruttner (1988), who considered it part of *A. m. carnica* in the western part of Romania and *A. m. macedonica* in the southeast. According to Foti (1965), a series of inter-zone morphological and behavioural differences would result in a series of ecotypes of *A. m. carpathica* in the West Plains, the Moldova Plateau, the Transylvania Plateau, and the mountain and steppe areas.

During the 1930s, a distinct population with yellow bands as *A. m. ligustica* was identified in Banat (the southwest part of the country). Based on other measurements, however, this population was in the range of *A. m. carnica* (e.g., Ruttner, 1988). At present, it seems that this "banat" bee is no longer found in the Banat area, and further research would be needed to identify any differences between these local bees from other populations and to support any conservation programme.

The Romanian breeding programme focuses on breeding "pure" Carpathian honey bees to preserve this national genetic resource. Despite this effort, some imports of other sub-species and hybrids (e.g., Buckfast) occurred during the past. This situation has been highlighted in several articles in Romanian beekeeping magazines in order to emphasize the importance of preserving native honey bees as they are naturally adapted to local conditions. Recent studies (unpublished data) performed at CNRS/LEGS (France) on 17 samples collected from different regions of Romania showed that 14 samples expressed the haplotype C1 (*A. m. carnica)*, 2 samples expressed the haplotype C2 (*A. m. ligustica*) and one samples show the differences

identified as the haplotype C3 (specific for Buckfast hybrids) This work used the COI –COII test, (Garnery *et al.*, 1993). In order to clarify the situation of the Romanian honey bee populations, further researches are necessary, using the modern tools of morphometrics and genetics, as well as studies regarding the adaptability at local ecosystems, comparing the results with data obtained from analogous studies on *A. m. carnica* and other European honey bee sub-species.

The morphometric studies are based on a standard procedure, as follows. One sample consists of about 30 honey bees per colony. The bees are taken from the edge frames of the bee colony in experimental cages. The bees are anaesthetized using chloroform and then placed in hot water in order to soften the chitinous parts and to extend the proboscis. Subsequently, bees are put into jars with Pampell fixative (i.e., 10 parts acetic acid, 20 parts formaline, 50 parts absolute ethanol, and 100 parts distilled water) for preservation and to remove the soft tissues. The proboscis, sternites, tergites III and IV, right metatarsus, right wings are detached, cleaned and put on microscopic slides, with 20-30 pieces per slide. Each slide is covered by another. Parts are measured with a binocular microscope (previously calibrated and provided with an ocular micrometer) or with a stereomicroscope provided with measurement software and video camera. The following measurements are made: proboscis length; fore and hind right wing length and width; cubital index; discoidal shift; number of hamuli; length and width of metatarsus; tergites III and IV width and colour; sternite III length, wax mirror length and width and distance between wax mirrors; and sternite VI length and width. Results from the measurements are shown in Tables 10 and 11.

Table 10. Results of morphometric measurements of 35 samples of honey bees collected from different regions of Romania in 2007. *measured from the beginning of the first cell.

Sample	Proboscis length (mm)	Cubital index	Forewing length (mm) *	Forewing width (mm)	Hind wing length (mm)	Hind wing width (mm)	Number of hamuli	Tarsian index
Average	6.3334	2.3174	8.748	3.129	6.335	1.879	21.5	1.75
Minimum	5.851	1.72	8.267	3.013	5.955	1.773	19	1.63
Maximum	6.773	3.02	9.094	3.271	6.622	1.983	24	1.88

Table 11. Results of morphometric measurements of 35 samples of honey bees collected from different Romanian regions in 2007. Note: Discoidal shift was positive for all samples.

Sample	Tergite III width (mm)	Tergite IV width (mm)	Sternite III length (mm)	Wax mirror width on sternite III (mm)	Wax mirror length on sternite III (mm)	The distance between wax mirrors (mm)	Sternite VI length (mm)	Sternite VI width (mm)
Average	2.277	2.261	2.677	1.402	2.346	0.376	2.674	3.158
Minimum	2.092	2.015	2.431	1.191	1.969	0.254	2.470	2.876
Maximum	2.501	2.593	2.900	1.556	2.603	0.725	3.614	3.369

Slovakia

In 2010 there were around 230,000 honey bee colonies (4.7 colonies / km^2) kept by 14,700 beekeepers in Slovakia (data obtained from the Central Registry of Beehives). Carniolan bees ("Slovakian" *carnica*) are the only sub-species known to exist in Slovakia, as importation of other sub-species is illegal. Morphometric methods are used to verify sub-species purity, and molecular and genetic methods for discrimination of ecotypes have recently begun to be used. The Slovakian lines of Carniolan bee actually in use are: Kosicanka; Vigor; Carnica Sokol; Hontianka; Vojnicanka; Tatranka and some non-registered lines. Four imported carniolan lines are allowed to be used in registered breeding stations, as well as for selling to beekeepers: Troiseck; Sklenar; Singer (Austria) and Vucko (Ukraine) (Kopernicky *et al*., 2005). In 2009, breeding is performed in four breeding and 35 reproduction stations; these registered stations have 2,500 colonies. There were 250 colonies in testing stations. The mission of the breeding stations is to breed and maintain their own lines of Carniolan bees and to supply this breeding material primarily to reproduction stations. The work of the breeding stations is focused mainly on keeping perfect breeding practices and not on the number of queens. Multiplying and selection of queens of registered lines from breeding stations to supply common apiaries is thus the role of the reproduction stations. The testing stations serve for anonymous verification of queen's offspring from breeding or reproduction stations. The testing of queens in these stations is based on data regarding the honey yield, the gentleness, the swarming tendency, the seating on combs, the development of the colony, the hygienic behaviour and tolerance to *V. destructor*. Each apiary in Slovakia is thus classified into one of four categories according to the level of breeding and selective works: breeding station, reproduction station, testing station or common apiary. The number of stations can vary from year to year according to re-evaluation or quarantine of some area due to American foulbrood infection. About 5,000 queens are produced in these stations annually; 200 queens are artificially inseminated.

Morphometric methods are used to verify sub-species purity in all registered stations (i.e. breeding, reproductive and testing stations), mainly based on measurements of cubital index, length of the proboscis; length and width of beeswings and colouring of first three abdominal tergites (which are sometimes yellowish). Sometimes also other morphometric characters are applied including number of hamuli, length and width of III and IV tergits, length and with of hind leg metatarsus and wax mirrors area. Parameters measured are then compared with limits appointed in the Standard for morphometric discrimination of "Slovakian" Carniolan bees (Kopernicky and Chlebo, 2004). Average values for the length of the proboscis are 6.68 mm and for the cubital index are 2.66. Race purity of Slovakian bee lines that belong to the *carnica* sub-species is confirmed also by use of DAWINO method. Probability ranges from 79 to 90% (Chlebo and Kopernicky, 2004).

Every registered station must keep one Slovakian line of Carniolan bee as a minimum. It is necessary to keep at least 40 and 30 colonies in the breeding and reproduction station respectively. At present (2009) there are 250 bee colonies (88 tested queens) in testing stations for queens of Slovakian lines of Carniolan bees. Queens are evaluated according to their progeny; at least four daughters are evaluated. Queens on testing stations are coded and evaluated anonymously. Colonies for testing of queens must be created before 15 September using nuclei or before 15 July using artificial swarms. Artificial insemination of breeding queens is used to cerate paternal and maternal colonies. The Institute of Apiculture inseminates and supplies pure bred queens. A project to discriminate bee populations in Slovakia using mtDNA analysis and characterisation of haplotypes is also underway.

Slovenia

Apis mellifera carnica is native to Slovenia and to some other regions of the former Yugoslavia, southern Austria, and parts of Hungary, Romania and Bulgaria (Ruttner, 1988). *A. m. carnica* expanded from its native range to the central and northern European countries, the USA, Canada and to other parts of the world through the practice of exportation, particularly during the second half of the 19th century. The main reasons for the widespread popularity of *A. m. carnica* are the gentle behaviour, good spring build-up of colonies and good summer honey production (Ruttner, 1992).

In 2004 the genetic variability of indigenous *A. m. carnica* was studied in Slovenia based on mitochondrial and nuclear DNA analyses (Susnik *et al.,* 2004). The main goal was to examine the purity or possible admixture of the Carniolan bee in the region. The results show that level of genetic variability within and among districts is low. All of the samples were fixed for one newly found mtDNA haplotype of the C phylogenetic lineage, designated as C2C. A low level of variability was observed for all microsatellite loci, showing a very homogenous structure of the Carniolan bee population. Samples collected in the neighbouring district of Croatia expressed very similar results. On the other hand, high genetic differentiation was observed in comparison with *A. m. macedonica*. The analyses confirm that the Carniolan bee from Slovenia represents an indigenous gene pool within the *A. m. carnica* population.

Besides molecular markers, the cubital index and colour of abdomen is used to distinguish native *A. m. carnica* from other sub-species. In our breeding programme for *A. m. carnica* the values of cubital index for workers must be 2.4 - 3.0, and 1.8 – 2.3 for drones. The colour of abdomen must be grey and not yellow. In the western part of the country our *A. m. carnica* is mixing with *A. m. ligustica.* These bees are easily recognised because of yellow abdomen. If there are more than 2% workers with yellow abdomen (in each colony) beekeepers must replace the queen. During next few years we plan to use data from mtDNA analysis in order to discriminate *A. m. carnica* from other honey bee populations and also to monitor which

haplotypes are reared from queen breeders.

Our recent studies of DNA variation (paper in preparation) has shown the diversity of populations and sub-species represented in breeding stations. Microsatellite DNA study has shown limited diversity in honey bee populations. The main neighbouring sub-species populations were considered and evaluated. The nature of 24 loci studied makes them suitable to compare and detect gene flow between the populations in and to derive the phylogenetic relationships between them. Honey bee populations have a limited level of genetic variation, considering studied loci. The isolated areas at different locations can limit the variability and on the other hand allow as to be effective in conserving available genetic diversity in the future breeding programme. The queen rearing practice and transportations of honey bee colonies over the country would not have significant effect on changes the genetic variability and allow us to perform the programme for conserving the native honey bee race. Importing queens and colonies of different genetic structure should not be allowed in order to reduce potential influx of foreign alleles and to allow us to conserve desired genetic diversity.

Spain

The honey bee sub-species of the Iberian Peninsula (Spain and Portugal) is *A. m. iberiensis* (Engel, 1999). This sub-species is closely related to the neighbouring *A. m. mellifera* occurring north of the Pyrenees and to *A. m. intermissa* at the north of the African continent. This relationship has been confirmed with morphological and molecular characters (see Cánovas *et al.*, 2008 and references therein), except with nuclear markers as the microsatellites (Franck *et al.*, 1998; Garnery *et al.*, 1998). In this sense Northern populations show *mellifera* mitochondrial DNA and those at the south *intermissa*-like following a northeast-southwest distribution gradient. Several attempts have failed to define ecotypes using morphometrics (Santiago *et al.*, 1986, but see Orantes-Bermejo and García-Fernández 1995). Only on islands are beekeepers interested in protecting their local ecotypes. The honey bee population on the Canary Islands has been subject to characterization and selection since 1996 (De la Rúa *et al.*, 1998, 2001). Particular mitochondrial haplotypes characterized the Canarian honey bee populations, lately confirmed as belonging to an Atlantic evolutionary sub-lineage of honey bee populations, also spread in other Macaronesian archipelagos such as the Azores and Madeira (De la Rúa *et al.*, 2006). Based on these results several regional laws established special measures to control the conservation, recuperation and selection of the Canary black honey bee in 2001. Among other activities, a natural mating area favoured by the particular topography of La Palma has been established allowing its saturation with local drones. The resulting mated local honey bee queens are distributed among beekeepers.

The methodology used to characterize the Iberian honey bee populations started with classical morphometric analyses (Cornuet and Fresnaye, 1989) and continued with molecular analyses (allozymes, pheromones, RLFP of the entire mtDNA molecule, microsatellites, SNPs, see De la Rúa *et al.*, 2009 for review) but the widespread *Dra*I test (Garnery *et al.*, 1993) is the method of choice to characterize the Iberian honey bee populations. With this method more than 25 different haplotypes have been detected in several studies (Miguel *et al.*, 2007; Cánovas *et al.*, 2008; De la Rúa *et al.*, 2009). These haplotypes defined by the restriction length polymorphism of a PCR amplified fragment of the intergenic region located between the $tRNA^{leu}$ and the cox2 genes, belonged to both the African (A) and the West European (M) evolutionary lineages. With rapidly decreasing costs for DNA analyses and automation we suggest the sequencing of the intergenic region whenever it is possible. In this sense Muñoz *et al.*, (2008) have demonstrated that the genetic diversity values increased over 1- to 5-fold when sequencing this region; for example the most distributed RFLP pattern in the Iberian Peninsula A2 corresponded to six different sequences in a sample of nine worker honey bees.

Other biparentally inherited nuclear markers that have been widely used to analyze the Iberian honey bees are microsatellites (Franck *et al.*, 1998; Garnery *et al.*, 1998; De la Rúa *et al.*, 2002; Miguel *et al.*, 2007). From their variation can be inferred population parameters such as heterozygosity values, Hardy-Weinberg and linkage equilibrium, effective population size, genetic distance matrix and neighbour-joining trees, providing powerful information about recent population events. The first microsatellites studies performed by Franck *et al.* (1998) and Garnery *et al.* (1998) gave full support to the taxonomy of the M lineage including the two sub-species *A. m. mellifera* and *A. m. iberiensis,* and confirmed the reduced genetic variability observed within the west European honey bee populations. On the other hand microsatellite data of unmanaged honey bee colonies from the south-east of the Iberian Peninsula related them to the African *A. m. intermissa*, although the presence of some alleles and the observed heterozygosity were characteristic of the European *A. m. mellifera*, that corroborated the postulated hybrid origin of *A. m. iberiensis* (De la Rúa *et al.,* 2002). In a recent study, three main factors postulated for sub-species differentiation between *A. m. mellifera* and *A. m. iberiensis* were the Pyrenean barrier, isolation by distance and the post-glacial re-colonization process (Miguel *et al.,* 2007).

To summarize the Iberian honey bee is one of the most studied European sub-species of *A. mellifera*, but all of these studies have led to the conclusion that in *A. m. iberiensis* populations there is a complex situation influenced by adaptation to local climatic conditions at a regional scale and the mobile beekeeping that has become a large-scale practice during the last decades.

Switzerland

In eastern Switzerland, where breeding programmes exist for both *A. m. mellifera* and *A. m. carnica*, the focus has mostly been on discrimination by cubital index, hantel index and discoidal shift, using paper charts and various computer programmes for the interpretation of the results (e.g. Coo-Recorder, Flügel-Index). Some breeders have used the DAWINO method (www.beedol.cz) or APMorph (www.apispro.de) including the coordinates of 19 and 18 vein intersections in forewings that compute 30 and 34 characteristics, respectively. These data are compared by a discriminant analysis method with known values of specified groups for single sub-species. Then the probability is computed by which the bee sample belongs to each of these groups. Although morphometric methods are quite suitable to distinguish between morphologically distinctly differentiated honey bee populations the overlapping of morphometric traits makes them unsuitable to reliably estimate admixture proportions. Furthermore, morphometric methods, especially wing measurements, focus on the distribution of those traits within a colony. It is assumed that cross-mating leads inevitably to a heterogenic distribution of phenotypic traits among patrilines within a colony, expressing a bimodal distribution. This is probably partly true in an environment where two different sub-species are newly admixed. It cannot be excluded, however, that some cross mating has remained undetected due to the overlapping distribution of the traits within the parental groups. During several decades of ongoing admixture and high selection pressure on several morphometric characters with a high heritability (Bienefeld, personal communication), the method has lost its informative power to detect hybridisation.

Modern molecular and population genetic methods have been developed to identify hybrids on an individual basis. These methods have widely been applied for scientific studies (De la Rúa *et al.*, 2002; Franck *et al.*, 2000; Garnery *et al.*, 1998; Jensen *et al.*, 2005; Soland-Reckeweg *et al.*, 2009) using neutral nuclear markers. The Swiss Bee Research Centre offers a service to identify hybrid individuals between different honey bee sub-species, using these modern molecular tools and statistical hybrid analysis methods (Soland-Reckeweg *et al.*, 2009). This service is aimed at breeders of *A. m. mellifera* and *A. m. carnica* using non-invasive queen sampling by harvesting 30 young drone larvae, and extracting their DNA together in one extraction mix.

An analysis method has been developed to identify hybrids between different honey bee sub-species using nine microsatellite loci and has been extended to 12 microsatellite loci (A007, A28, A43, Ap33, Ap289, Ap273, Ap226, Ac306, Ap1, A29, A76, B24 (Solignac *et al.*, 2003). To test for hybridisation on an individual basis, a Bayesian framework of model-based clustering is used (Anderson and Thompson, 2002). This method was specifically developed for the identification of hybrid individuals in a potentially admixed population. It is implemented in the programme "NewHybrids 1.1 beta" and assumes that hybridisation could have arisen during two generations. It estimates the probability of an individual to belong to one of six different hybrid classes (pure 1, pure 2, F1 = pure 1 x pure 2, F2 = F1 x F1, backcross 1 = F1 x pure 1, backcross 2 = F1 x pure 2) (Fig. 5). The contribution of one group to the hybrid classes are the following; pure 1 and 2 = 100% and 0%, respectively, F1 and F2 = 50%; backcross 1 and 2 = 75% and 25%, respectively. Computations are run for each individual separately using pure reference samples for *A.m. mellifera* from Switzerland, Norway and France and for *A.m. carnica*

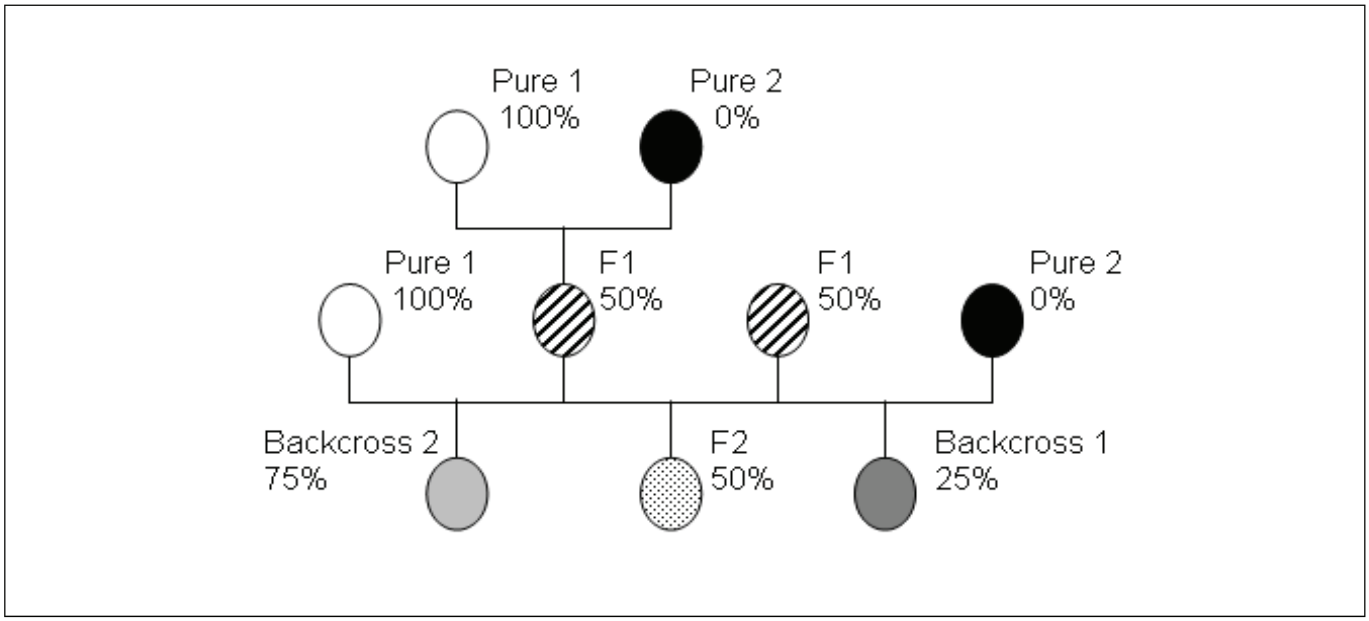

Fig. 5. Diagram of the hybridisation model and the relative genetic contribution of the different hybrid classes with respect to sub-species "pure 1" used in Switzerland.

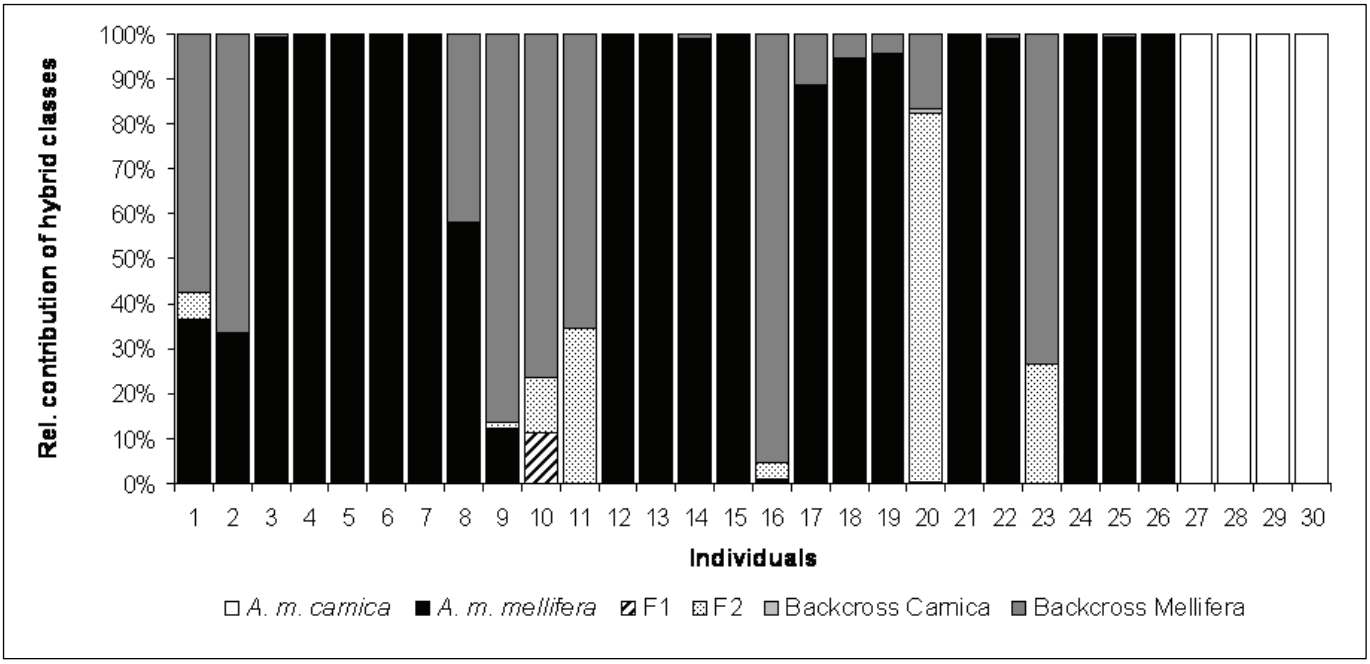

Fig. 6. Diagram of the relative contribution of individual hybrid classes for 30 individuals, tested in 2008. Individuals 1-26 originate from a Swiss *A. m. mellifera* breeding population. Individuals 27-30 originate from an *A. m. carnica* population from Austria. 15 individuals (3-7, 12-15, 19, 21, 22 and 24-26) have a probability of > 0.95 to belong to hybrid class "pure *A. m. mellifera*". 4 individuals (27-30) have a probability of p> 0.95 to belong to hybrid class "pure *A. m. carnica*". 8 individuals (1, 2, 8-11, 16-18 and 23) have a probability of p>0.50 to belong to hybrid class "backcross with *A. m. mellifera*". One individual (20) has a probability of 0.82 to belong to hybrid class F2.

from Slovenia and Austria (Soland-Reckeweg *et al.,* 2009). Besides the reference populations, no prior information about sub-species, population or group associations are used. The threshold for hybrid individuals is set to $p < 0.95$ within the hybrid class "pure" in order to reduce the misclassification of backcrossed individuals as pure to a minimum (Vähä and Primmer, 2006). Individuals that could not be classified as "pure" are classified within the class with the highest probability. Fig. 6 shows an example for different pure *A. m. mellifera*, *A. m. carnica* and individuals with various probabilities within the class "backcross with *A. m. mellifera*". An individual with a probability of $p < 0.95$ in class "pure" is automatically assigned to the respective hybrid class with the highest probability, even if the probability in this class is smaller than the probability to be "pure" (e.g. Fig. 6; Individuals 8, 17, 18). This threshold takes into account the limitation of the method that produces lower probabilities in case of more ancient hybrid events, as this does not match the model assumption. The aim is to clearly identify pure individuals compared to hybrids or individuals of unclear status. It is likely that a probability of $p > 5\%$ in "backcross" reflects a more ancient hybrid event with reoccurring backcrossing with pure individuals. This interpretation is supported by the fact that all individuals of the reference samples have a probability of $p \geq 0.98$ to be pure members of their respective sub-species (Soland-Reckeweg *et al.*, 2009). As the Swiss *A. m. mellifera* population shows a high degree of hybridisation especially within the class "backcrosses with *A. m. mellifera*" one would expect to obtain various "degrees" of backcrossing, as over time, hybrids are crossing with pure and other backcrossed individuals, regaining a higher and higher probability to be "pure".

Although the method is very robust due to the clear differentiation between *A. m. mellifera* and *A. m. carnica* and *A. m. ligustica*, it only poorly differentiates *A. m. carnica* and *A. m. ligustica* themselves (Franck *et al.*, 2000; Soland-Reckeweg *et al.*, 2009). The method therefore mainly applies to breeding stocks of *A. m. mellifera* and *A.m. carnica* where hybridisation between these two sub-species is likely to occur.

A total of 141 individual honey bees were analysed in 2007 and 2008. Samples originated from breeding programmes for *A. m. mellifera* and *A. m. carnica* in Switzerland and Austria. Overall, 18 queens were identified as pure *A. m. carnica* and 83 as pure *A. m. mellifera*. From the 40 hybrids, 37 could be identified as backcrosses of F1 hybrids with pure *A. m. mellifera*. Only three individuals were of recent hybrid origin, being F2 hybrids (cross between two F1 hybrids). No F1 hybrids could be identified at all. Generally, all of the hybrids originated from an *A. m. mellifera* population. Besides the three F2 hybrids, all but one of the *A. m. mellifera* and all of the *A. m. carnica* queens could be assigned to their respective sub-species.

The high relative amount of hybrids within *A. m. mellifera* clearly shows that the wing measurements that have been performed by the breeders over decades do not seem to have had the desired effect. On the contrary, the large amount of backcrossed individuals suggests, that a few reoccurring hybridisation events remained undetected and that the descendants from these crossings have been

Table 12. Occurrence of pure and hybrid classes in two consecutive years of testing from individual queens from an *A. m. mellifera* population in Switzerland. The frequency of pure *A. m. mellifera* queens increased significantly paralleled by a decrease of hybrids (X^2 = 576.88, p ≤ 0.001).

Year	Pure		Hybrids		Total	
	No	%	No	%	No	%
2007	28	53	25	47	53	100
2008	53	78	15	22	68	100
Total	81	67	40	33	121	100

prolific within the breeding population. This observation is in agreement with the results of a previous scientific study by Soland-Reckeweg *et al.* (2009).

None of the *A. m. carnica* individuals showed any sign of hybridisation. It is likely that this is a result from the area wide queen change in western Switzerland, where most of the tested *A. m. carnica* samples originated. The *A. m. mellifera* samples, on the other hand, originate from an area where they occur in sympatry with *A. m. carnica*. The advantage of this method is therefore mainly to *A. m. mellifera* and *A. m .carnica* populations from admixed areas.

The success of the molecular testing for purity is shown in Table 12. In the second year there was a significant increase in the number of pure queens, paralleled by a decrease of hybrids. The decrease of hybrid *vs.* pure queens shows that the use of molecular tools is highly efficient to obtain pure breeding stock after generations of reoccurring hybridisation. This could be especially useful in the management of conservation areas or the maintenance of threatened sub-species like *A. m. mellifera* or *A. m. sicula.*

Due to the relatively high costs it is not possible to test a large number of worker bees as with morphometric measuring, so molecular testing only reveals the hybrid status of the queen, not of her daughters. It is therefore of high importance that breeders are given the opportunity to use mating apiaries with molecularly tested, drone-producing queens. Daughter queens that are mated in these yards are molecularly tested again and if considered pure, the granddaughter generation can safely be produced as pure queens. This implies that the mating yards are sufficiently isolated. So far, molecular testing has officially been adopted by two breeding programmes in Switzerland, one for *A. m. mellifera* and one for *A. m. carnica*. The cost for the testing the mother queens is generally added to the price of daughters produced by these queens, and that for the testing of drone producing colonies is mainly covered by the respective breeding community.

Making molecular testing freely available to bee breeders has greatly improved the confidence in their respective breeding stock. This has led to a revaluation of priorities, as in most pure breeding communities the cubital index was of highest importance to breeders. Breeders now focus much more on the performance of their colonies as they are given security about the result of the purity testing.

Turkey

Anatolia, the Asian part of Turkey, has played an important role in the evolution of honey bees for a number of reasons. Firstly due to the diverse climatic conditions, with a diverse geological and topographical structure varying from region to region and which was a refuge of glaciers. Anatolia forms a natural bridge between Asia, Africa, and Europe where different honey bee races dispersed, exchanged genes, and then adapted to those different climatic and floristic conditions. Beekeeping activity has been recorded in Anatolia since 1300 BC according to tablets made by the Hittites (Bodenheimer, 1942). Turkey has the largest number of colonies in the world after China, with 4.8 million hives (Department of Statistics of Turkey). Honey bees have found opportunities to adapt to a diversity of ecological conditions that include three phytogeographic regions: Euro -Syberian; Mediterranean; and Irano-Turanian. Interactions between bees and the local floral characteristics have led to morphological, biochemical, physiological, and behavioural adaptations to form several honey bee ecotypes. For the last fifteen years, we have studied the diversity of Turkish honey bees. We studied morphometric variation in honey bee workers to demonstrate the differentiation within honey bee populations. Since the conditions within hives are more or less constant, we would expect phenotypic differences to largely reflect genetic differences. The classification and discrimination of the world honey bee sub-species of Ruttner (1988) using morphometric analyses is still largely valid.

Allozymes, different forms of the same enzyme protein coded by the different alleles of the same gene, have been used quite extensively in studying the genetic diversity of organisms. Although variation in allozyme frequencies has been proven to be very useful in studying genetic diversity, it has the drawback that allozyme loci are not generally selectively neutral so that phylogenetic relationships obtained from these loci may not reflect the true phylogeny (Futuyma, 2009). Furthermore, in honey bees there is not much variation in allozymes, probably due to the haplodiploidy of honey bees (Lester and Selander, 1979). Nevertheless, allozyme studies can still show genetic differentiation amongst honey bee sub-species and populations.

The study of variation in DNA has shown a great utility in demonstrating the diversity of populations and sub-species. Microsatellite DNA and RAPD studies in our laboratory have shown a great diversity in honey bee populations of Turkey (Bodur *et al.,* 2007; Tunca *et al.*, 2009). In general the neutral nature of these loci makes them very suitable to study the differentiation and gene flow between the populations and to derive the phylogenetic relationships between them.

In Anatolia there are four honey bee sub-species according to multivariate analysis of morphometric data (Kandemir *et al.*, 2000). These are *A. m. anatoliaca, A. m. caucasica, A. m. meda,* and *A. m. syriaca,* which were considered by Ruttner (1988) to form the morphological branch O. Another sub-species, belonging to the "*carnica*" group is distributed in Thrace, the European part of Turkey, and belongs to mitochondrial C lineage. In addition to those sub-species there are ecotypes that are less well defined and need further investigation. Among them are Mugla bees, Yıgılca bees, and Giresun bees. For example, the Mugla bee is an ecotype of *A. m. anatoliaca* which has a quite different life history adapted to foraging on the scale insect *Marchalleina hellenica* on pine trees, so that it continues to produce brood to build up a large population in the autumn while Anatolian bees in other regions stop producing brood in order to prepare for winter (Dogaroglu, 2009). Yıgılca and Giresun bees are also considered to be ecotypes of *A. m. anatoliaca*, discriminated from it by larger size and darker colour respectively.

Methodology used for discrimination of honey bee populations in Turkey includes morphology, behaviour, classical morphometric analysis, geometric morphometrics, allozymes, mtDNA, RAPD, microsatellites. Inter SSR and SNP analyses are also underway.

Classical morphometric analysis

This analysis is based on a number of morphometric characters of wings and legs (Kandemir *et al.*, 2000). The wings and legs were dissected from bees and mounted on microscope slides which were photographed under a Leica stereo microscope and images were transmitted to a computer**.** Measurements are analyzed by multivariate statistics. In one of these studies 10 morphometric characters were measured on the forewing and hindlegs on 2,905 worker bees collected from 383 hives in 36 provinces analyzed by SYNTAX V (Podani, 1993) and a UPGMA phenogram was constructed based on Mahalanobis distances calculated using NTSYS (Rohlf, 1992). Two major groups were found by discriminant function analysis; the first group included only bees from Thrace region where *A. m. carnica* type bees are found, the second group consisted of Anatolian bees.

In another study we analyzed 10 honey bee populations in a geography ranging from Austria to Nakhcivan (Fig. 7) based on 10 morphometric traits from wings and legs, Austria and Kırklareli were found within the same cluster which indicates relationship of the Kirklareli ecotype to *A. m. carnica*. The second cluster consisted of Artvin, Ardahan, Kars, and Igdır, and these represented *A. m. caucasica* bees. The third cluster belonged to Nakhcivan representing İranian bees; *A. m. meda* and an Ankara population formed the fourth cluster which represents *A. m. anatoliaca* (Fig. 8) (Kandemir *et al.*, 2005). The best discriminating characters were the length and width of forewing, angles on the wing venation, distance c, distance d, and the metatarsus width. Cubital index clearly separates the Kirklareli ecotype (2.72) from the rest of the sub-species of Turkish honey bees and place it close to *A. m. carnica* (2.78).

Geometric morphometrics

Geometric morphometrics provide a powerful tool to quantify the overall shape variation among organisms independent of size, based on a number of landmarks (homologous structures on the body of

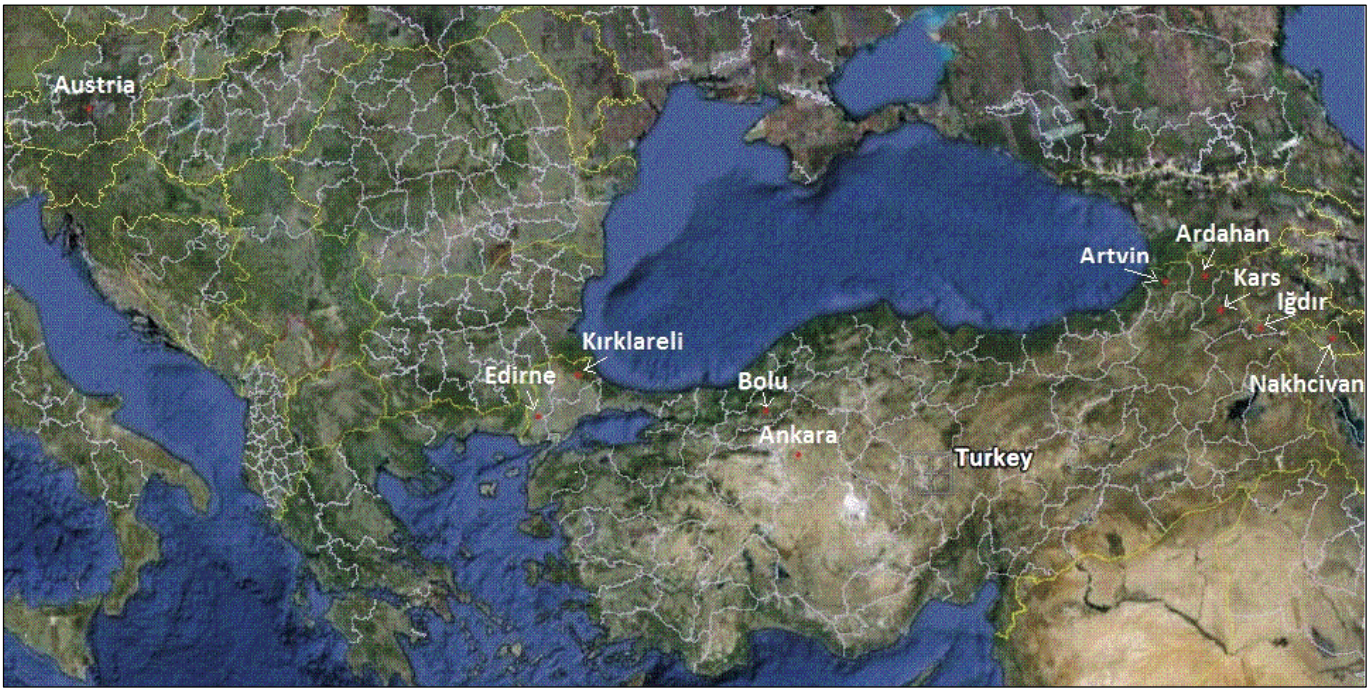

Fig. 7. Map of the locations in the Turkey region, ranging from Austria to Nakhcivan bees, sampled and used in analysis of ten honey bee populations based on 10 morphometric traits from wings and legs. Results of analysis are shown in Fig. 8.

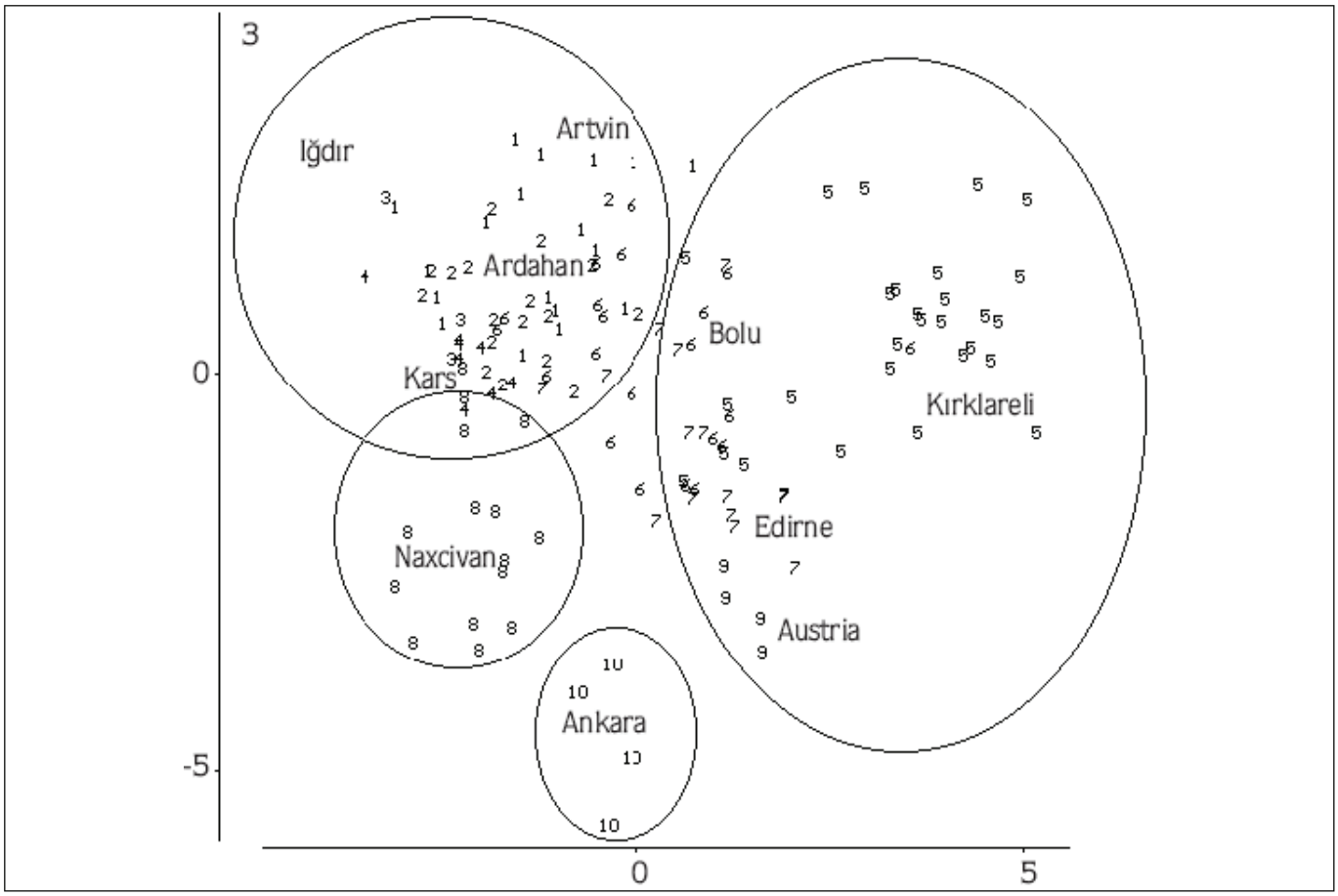

Fig. 8. Discriminant Function Analysis of honey bee populations from Turkey, Naxcivan (also spelt Nakhciva, Azerbaijan), and Austria based on morphometric data (Kandemir *et al.*, 2005).

specimens studied). Nineteen different landmarks on 574 honey bee samples collected from 10 regions in Turkey were used for geometric morphometry analyses. Tps dig version 2.05 (Rohlf, 2006), Morpheus (Slice, 1998), and IMP software were used for geometric morphometry analysis. Data obtained are used to discriminate between the groups by Canonical Variate Analysis. The honey bee sub -species are well separated on the basis of shape which largely reflects genetic differences (Kence *et al.*, 2009).

Allozymes

Allozymes of Turkish honey bees have been studied extensively at the Middle East Technical University (Kandemir and Kence, 1995; Gencler, 1998; Hadimogulları, 2002; Kandemir *et al.*, 2000, 2006). In the most extensive study, honey bees were sampled from 77 localities within 36 provinces in Turkey (Kandemir *et al.*, 2000). Six enzyme loci were studied in a total of nearly 3,000 bees and four of them: esterase (*EST*); phosphoglucomutase (*PGM*); malatedehydrogenase (*MDH*); and hexokinase (*HK*) were polymorphic. *PGM* showed the highest polymorphism among the loci studied, having five different alleles. This is the highest allelic diversity reported worldwide. *MDH* also had a very high allelic diversity with five alleles, but with less heterozygosity compared to *PGM*. MDH^{100} is the most common allele in honey bee populations of Turkey with a frequency of almost 1.00 in Anatolia. The frequency of MDH^{100} drops in Thrace to 0.64, and the frequency of MDH^{65} goes up to 0.36. These alleles and their frequencies markedly separate European and Anatolian honey bees, also indicating the closeness of the Kirklareli ecotype to *A. m. carnica*. The presence of allele MDH^{116} distinguishes *A. m. caucasica* from in the rest of Anatolia and in Thrace (Fig. 9).

mtDNA

We have studied mtDNA variation in Turkish honey bees. *Dra*I restriction analysis of the *COI-COII* intergenic region yielded seven haplotypes. Haplotype 1 was the most common in Turkey: haplotype 2 was distributed in Eastern Anatolia. Mitochondrial ND2 sequence analysis clustered Hatay bees with *A.m. lamarckii* and *A.m. meda*, while bees from central Anatolia clustered within the C morphometrical lineage (Kandemir *et al.*, 2006). COI-COII region sequence analysis revealed 12 different mitotypes in honey bee populations of Turkey (Solorzano *et al.,* 2009); some are unique to Artvin, Giresun, and Kirklareli.

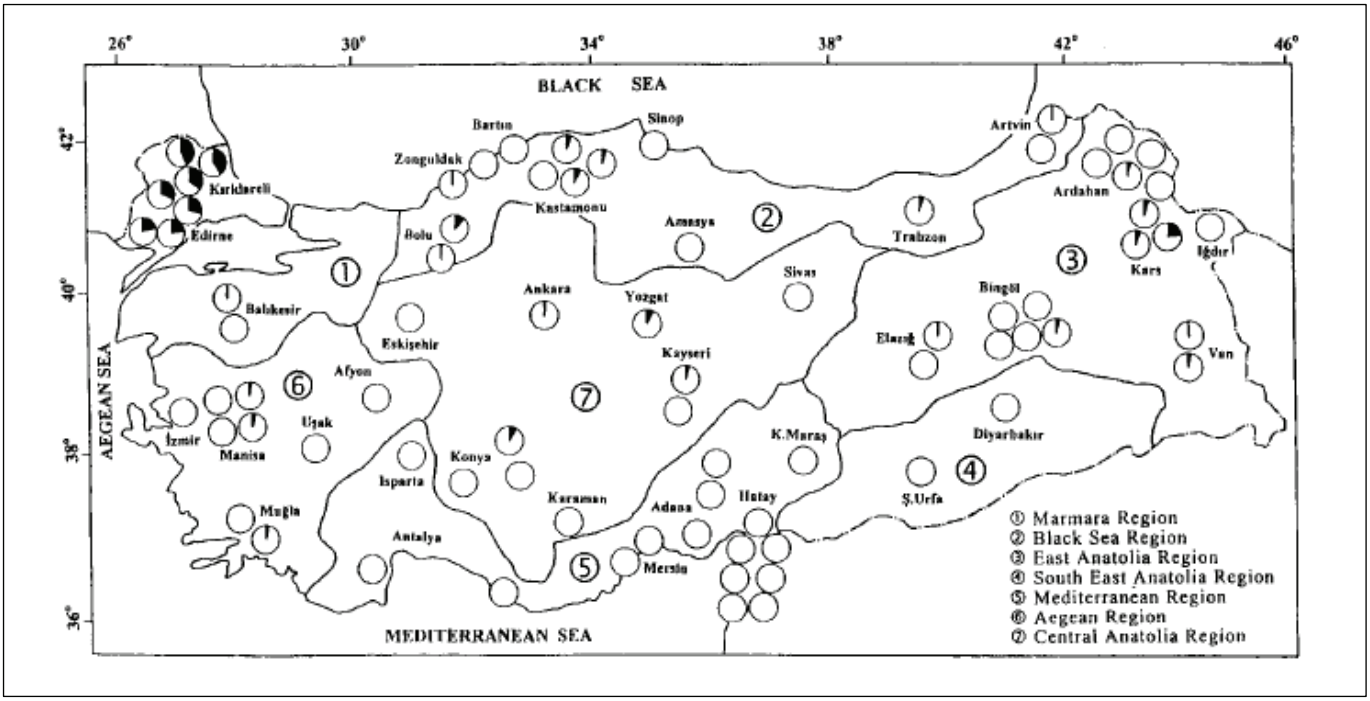

Fig. 9. The distribution of *MDH* allele frequencies in honey bee populations of Turkey (Kandemir *et al.*, 2000). MDH^{100} allele frequencies are high, fixed in most of the Anatolian populations, whereas in Thrace MDH^{65} is found in higher frequencies discriminating the bees of two regions.

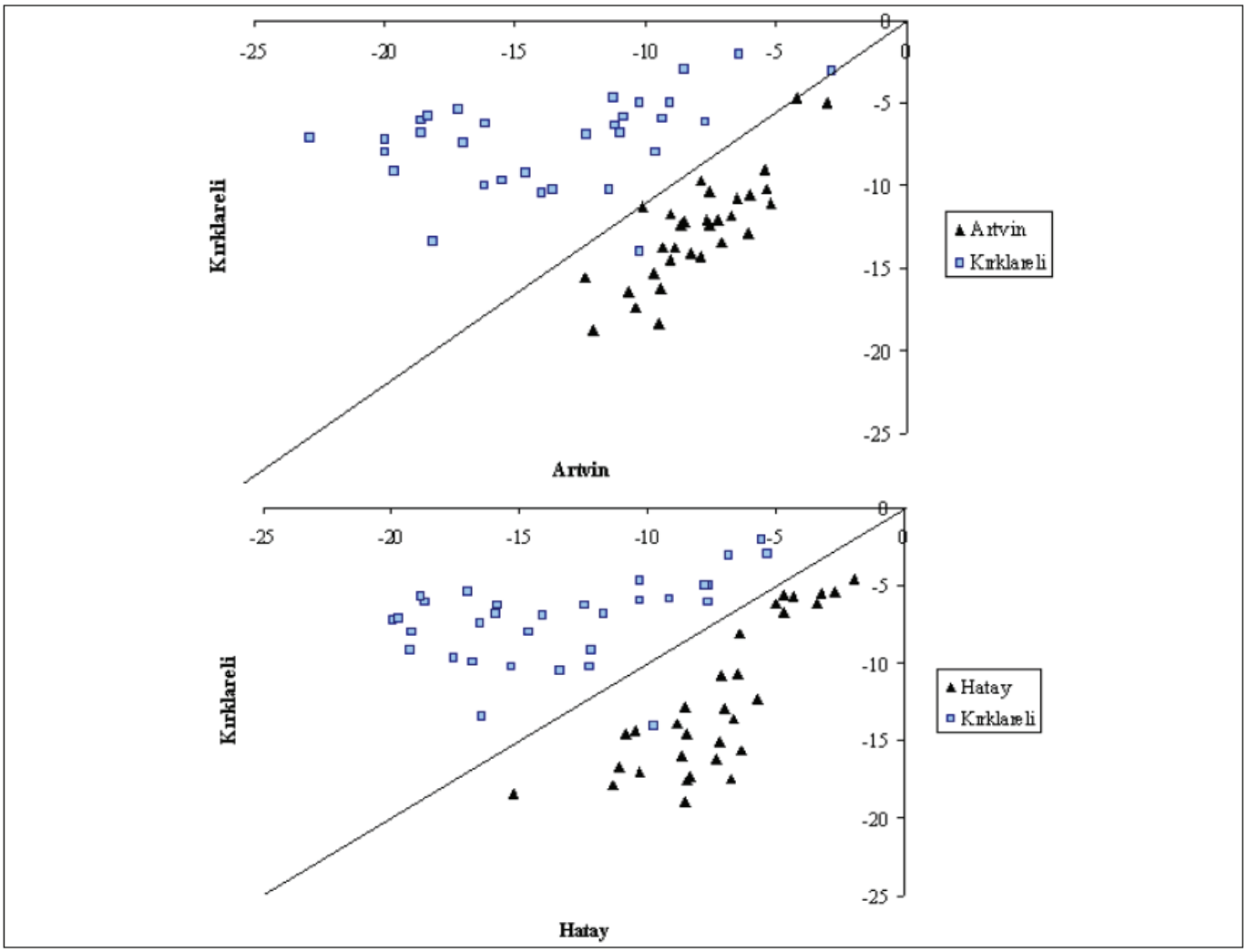

Fig. 10. Loglikelihood plots of Turkish individuals for population-pairs to be discriminated (Bodur *et al.*, 2007). High assignment percentages indicate that the individuals sampled from one population are similar to each other, yet different from others.

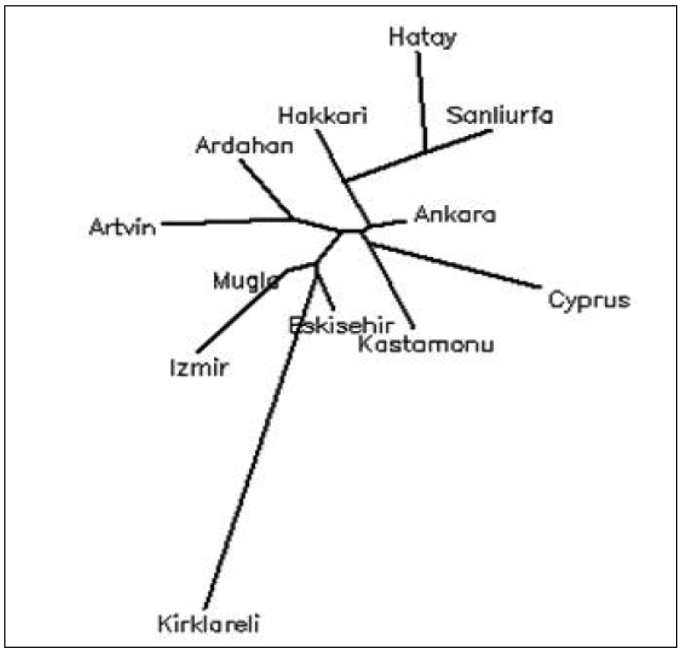

Fig. 11. Unrooted Neighbour-Joining tree of the 12 populations sampled in Turkey and Cyprus based on D_{LR} distance calculated from microsatellite data (Bodur *et al.*, 2007).

Randomly Amplified Polymorphic DNA (RAPD) analysis

In a recent investigation (Tunca *et al.*, 2009), 10 RAPD markers were used to assess genetic diversity. Neighbour-joining analysis showed that the RAPD markers successfully discriminated between honey bees of the A and C lineages.

Microsatellites

Bodur *et al.* (2007) studied the genetic variation and differentiation in twelve honey bee populations from Turkey and Cyprus (Figs 10, 11) with nine microsatellite loci (A7, A24, A28, A43, A113, Ap43, Ap68, Ap226, and Ac306). A total of 165 alleles, (6.83 mean numbers of alleles per locus per population) were found. Six alleles at three loci were recorded for the first time; four alleles at three loci were recorded previously only in Africa. Kırklareli, İzmir, Kastamonu, Cyprus, and Artvin had the lowest Nm values (rate of the gene flow), (< 2) which indicates that there is no significant gene flow to these populations. The estimated pairwise F_{ST} values ranged between 0.02 and 0.183.

Assignment tests

Assignment tests were performed too see the likelihoods of individuals belonging to different honey bee populations. Percentages of correct assignments are the percentages of the individuals that are correctly assigned to the population from which they were sampled. Populations of Ardahan, Artvin, Cyprus, Izmir, Kirklareli, and Urfa were found to have correct assignment percentages higher than 70%, which indicates a high level of genetic differentiation at these populations in agreement with the high F_{ST} and low Nm scores. Individuals from Artvin and Kirklareli are assigned correctly with 71% and 87% respectively. These populations are genetically isolated, and are under protection in those areas.

In conclusion, Turkish honey bee populations have a high level of genetic variation, illustrating the role of Anatolia as the genetic centre of Middle East honey bees. The isolated areas at Artvin, Ardahan, and Kirklareli seem to be effectively conserving genetic diversity. We recommend that the practice of rearing and distributing honey bee queens from a few localities to all over the country and importing honey bees from abroad should be stopped as an immediate precaution in order to be able to conserve honey bee genetic diversity. We conclude that lineage determinations should not rely only on mtDNA, and that other traits such as the polymorphism in allozymes and microsatellites and SNPs should also be studied in order to support the classification and discrimination derived from morphometric analyses.

The United Kingdom and the Republic of Ireland

In recent years, an inexplicable belief has arisen amongst some conservation professionals that the honey bee is not native to the British Isles. Archaeological evidence including honey bees preserved in Bronze Age (*c.* 2000-1700 BC) deposits, and honey bees sufficiently well preserved to permit wing morphometric analysis from Anglo-Scandinavian (Viking) deposits (*c.* 935-975 AD), has, however, proved beyond all reasonable doubt that the honey bee is indeed native to the British Isles, and that the native sub-species was the Dark European honey bee *A. m. mellifera* (see review in Carreck, 2008), known as the British (or English Irish, Scottish or Welsh) "black bee".

Since the middle of the 19th century, however, extensive imports of other strains of honey bee, principally *A. m. ligustica* and *A. m. carnica*, in to the British Isles took place. Disastrous colony losses occurred throughout the British Isles in the early 20th century due to the "Isle of Wight Disease", at the time thought to be due to the tracheal mite *Acarapis woodi*, but now thought to have been due to a combination of factors, including viruses (Neumann and Carreck, 2010). Extensive queen imports took place in a desire to restock, partly prompted by the belief that the native bee was particularly prone to disease. Brother Adam, of Buckfast Abbey, Devon, perpetuated this idea, and promoted his own "Buckfast" strain of hybrid bee (Adam, 1968). It therefore became an accepted "fact" that *A. m. mellifera* was extinct in the British Isles.

In the years following the Second World War, however, a number of beekeepers became dissatisfied with the mongrel bees that they were using. Led by the late Beowulf Cooper, a professional entomologist, they formed what is now the Bee Improvement and Bee Breeders Association (BIBBA). They soon concluded, using wing morphometric studies, that in many areas, "near-native" dark bees, apparently of *A. m. mellifera*, still existed and had desirable characteristics suitable for the climate and available forage (Cooper, 1986).

To date, the only significant use of molecular techniques to study British honey bees has been as part of the EU funded project "Beekeeping and Apis Biodiversity in Europe" (BABE). These limited studies confirmed that *A. m. mellifera* does indeed still exist. Samples from England, Ireland, and Scotland were found to be closely related to samples collected from France, and Læsø, Denmark, as well as to samples from Tasmania, Australia, known to have been descended from bees imported from England in the 19th Century (Jensen and Pederson, 2005). Intriguingly, two other samples from the east coast of England were found to be indistinguishable from samples from Norway and Sweden, raising the possibility that Viking settlers in the 10th century brought honey bees with them. BIBBA is currently organising an ambitions "Project Discovery" which aims to collect bee samples from throughout the British Isles for molecular studies to provide information on their origins.

It is clear, however, that most bees, especially in the south east of England are mongrels, derived from many races, and individual beekeepers champion different strains. Ironically, despite its origin in England and popularity worldwide, the Buckfast bee has not been commercially available in Britain for many years, so is not widely used. In the remoter parts of south west and northern England, and Wales, Scotland and Ireland, the bees tend to be dark, and of "near-native" type.

There never has been any state funded bee breeding or queen rearing programme in the British Isles, so bee breeding is on a small scale, mainly run by amateur beekeeping groups. A very small number of commercial beekeepers sell a few thousand reared queens a year, and some thousands more queens of various sub-species are imported from abroad each year, but it is clear that the vast majority of the 250,000 or so honey bee colonies in the British Isles are allowed to requeen themselves, with no selection or control of mating.

In recent years, however, there has been a resurgence of interest in conserving and developing *A. m. mellifera*, and a number of active breeding groups, now operate under the auspices of BIBBA in England, Ireland, Scotland and Wales, but the number of queens raised each year is small. In addition, a number of small breeding programmes have recently been established to attempt to breed bees tolerant to infestation by *V. destructor*, and these include the programme at the University of Sussex, using *A. m. mellifera* (Carreck *et al*., 2010).

Potential new methods for discrimination of honey bee populations

As discussed previously, polymorphisms at the DNA level enable the use of appropriate molecular markers for discriminating honey bee sub-species and populations. Recently, DNA Barcoding has been developed to create a standardized, cost- and time-effective, method to identify species. The DNA Barcode consists of a short mitochondrial genetic marker, which can distinguish individuals of a species based on the principle that genetic variation between species exceeds the variation within species (Hebert *et al*., 2003; Hebert *et al.,* 2004). The "Barcode of Life" project aims to develop DNA barcoding for the identification of species using a standard region of the cytochrome c oxidase subunit 1 and create a public and interactive reference database where the sequences of all specimens can be deposited (http://www.barcodinglife.org). Despite some criticisms about the validity of this single method to identify species (Meyer and Paulay, 2005; Rubinoff *et al.,* 2006), the number of DNA barcode sequences increases, and with the addition of information on geographic distribution and genetic diversity and structure, DNA barcoding is expected to improve molecular phylogenetics and population genetics studies (Hajibabaei *et al.,* 2007). Different honey bee species have been already barcoded, but it is not yet known whether it would be possible to discriminate between honey bee populations using this method.

Thanks to high-throughput genotyping technology, a new generation of molecular markers, called single nucleotide polymorphisms (SNPs), are available (Vignal *et al.,* 2002). SNPs, which are single base changes in the DNA sequence, represent powerful markers for evolutionary and population genetics studies. Indeed, with the publication of the honey bee genome, they have been used to study at the genome level the geographical structure of honey bee sub-species (Whitfield *et al.,* 2006) and the evolution of African-derived bees (Zayed and Whitfield, 2008). SNP markers can thus allow the discrimination of honey bee populations and sub-species. In addition, because genome-wide SNPs promise to be important in livestock breeding programmes (Zukowski *et al.,* 2009) and for the identification of genetic association with diseases (Gibbs and Singleton, 2006), they have the potential to contribute to honey bee selection based on resistance to environmental stressors.

Conclusions and perspectives

In this review paper, the methodology used for the discrimination of honey bee populations in 19 different European countries has been presented, together with the results from each method. The approaches have been summarized for morphometric analysis (classical and geometric) and for different molecular techniques. Even if the same approach is used by different laboratories, different techniques for making measurements, different morphometric landmarks, molecular markers or statistical procedures may be used, and these variations lead to results that cannot be directly compared between countries. It is therefore necessary to use common methods in all countries, and therefore one of the main goals of Working Group 4 (WG4) of the global "Prevention of honey bee COLony LOSSes" (COLOSS) Network is to establish common protocols for the discrimination of

honey bee populations. Discussion is currently taking place between the scientists of WG4 about the protocols that should be used in the future. We hope that soon these common protocols will be published and help beekeepers to establish breeding programmes that would be founded on well-characterized populations.

Acknowledgements

COLOSS is financially supported by COST via Action FA0803. We would like to thank the referees for their useful, important and detailed comments. We would also like to thank Aphrodite Dimopoulou, PhD student in English drama, for corrections to the language.

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