ORIGINAL RESEARCH ARTICLE

Comparison of microsatellite DNA diversity among commercial queen breeder stocks of Italian honey bees in the United States and Italy.

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Summary

Declines in the numbers of breeder honey bee queens and the concomitant loss of genetic diversity could potentially result in inbreeding, and increased susceptibility to pests and diseases. Genetic diversity of commercial Italian bee colonies in the United States and Italy was assessed using six variable microsatellite DNA loci. Worker bees were sampled from colonies of queen breeders in both countries (USA, n = 18; Italy, n = 24). Overall, allelic richness (mean alleles/locus), gene diversity (heterozygosity), and F_{IS} (inbreeding coefficient) did not differ between the two groups. A total of 48 alleles were present among all colonies. Sampled colonies from each country had a total of 38 alleles, although alleles were present that were unique to each group. There were a total of 10 unique alleles among USA bees and 10 among Italian bees. Estimates of the level of genetic differentiation based on different allele frequency patterns among the USA and Italian bees were measured with the population genetic parameter F_{ST}. These estimates showed that bees from the USA and Italy were measurably distinct relative to the frequencies of the microsatellite alleles present in samples from each country. Overall allelic diversity levels were sufficiently high indicating that inbreeding does not appear to be an immediate threat to existing honey bee populations.

Comparación de la diversidad de microsatélites de ADN entre ganado de abejas italianas procedentes de criadores de reinas comerciales de los Estados Unidos y de Italia.

Resumen

El descenso del número de criadores de abejas reinas y la concomitante pérdida de diversidad genética puede resultar potencialmente en endogamia y en un aumento de la susceptibilidad a plagas y enfermedades. La diversidad genética de colonias comerciales de abejas italianas en los Estados Unidos y en Italia fue evaluada usando seis loci variables de microsatélites de ADN. Abejas obreras fueron muestreadas de colonias de criadores de reinas en ambos países (USA, n = 18; Italia, n = 24). En general, la riqueza alélica (media de alelos por locus), la diversidad génica (heterocigosidad) y F_{IS} (coeficiente de entrecruzamiento) no fueron diferentes entre los dos grupos. Un total de 48 alelos se observaron en el conjunto de todas las colonias. Las colonias muestreadas en cada país mostraron un total de 38 alelos, aunque algunos alelos aparecieron exclusivamente en cada grupo. Hubo un total de 10 alelos únicos en las abejas de USA y otros 10 en las italianas. Las estimaciones del nivel de diferenciación génica basadas en patrones de frecuencia de alelos entre las abejas de USA y de Italia fueron medidas con el parámetro de genética de poblaciones F_{ST}. Estas estimaciones mostraron que las abejas de USA y de Italia son cuantitativamente distintas en relación a las frecuencias de alelos de microsatélites presentes en las muestras de cada país. En general, los niveles de diversidad de alelos fueron suficientemente altos, indicando que el entrecruzamiento no parece ser un peligro inmediato para las poblaciones existentes de abejas.

Keywords: honey bee, *Varroa destructor*, microsatellite, genetic diversity, Italy, Italian bees

Introduction

Italian bees, *Apis mellifera ligustica*, have long been the most popular race of honey bee sold in the United States (Ruttner, 1975). Importation of bees into the USA from Italy persisted from 1859 (Sheppard, 1989) until 1922 when an Act of the USA Congress terminated importation of Italian honey bee stock as a preventative measure for the further spread of the tracheal mite, *Acarapis woodi* (Cale, 1922). This effectively eliminated the potential for the addition of new alleles into USA populations of Italian bees through gene flow, and put the USA population at risk of eventual inbreeding. Inbreeding was not thought to be a major concern, as support for continued restrictions on the importation of Italian bees into the USA was based on studies showing sufficient genetic diversity in existing populations to support continued breeding efforts (Tucker, 1976; Rinderer and Harbo, 1983).

Previous studies assessing allozyme variation among queen breeding operations across the USA determined that differentiation was evident for apiaries in California and the south east (Schiff and Sheppard, 1995; 1996). One potential contributing factor to the differentiation measured in the Schiff and Sheppard (1995; 1996) studies is that the largest proportion of queen breeders are located in California. While breeders do perform outcrossing to maintain genetic diversity, a limited gene pool of queens could potentially affect overall genetic diversity in the USA beekeeping industry.

A reduction in the number of breeder queens, from disease outbreak or other catastrophic events, could result in a severe genetic bottleneck. This could lead to inbreeding and a reduction in genetic diversity, resulting in elevated levels of susceptibility to pests and diseases and a general decrease in the quality of commercial honey bee stocks. Concerns about possible inbreeding in honey bee populations in the USA have arisen owing to the overall decrease in colony numbers from over 5 million to 2.4 million (National Research Council, 2007) and also the limited sources of queens from queen breeders. The notable decline in pollinators in the USA in recent years is an important issue. The role that genetic diversity has played in such declines is unknown and should be addressed.

We sampled genetic diversity among colonies from queen breeders representing Italian (*A. m. ligustica*) stock in both the USA and Italy to investigate the genetic potential of current USA breeder queens and to assess if there has been any loss of diversity among the USA population relative to breeder queens in Italy. Significant loss of genetic diversity among commercial stocks of bees would have potential implications for the need for integration of new honey bees (i.e., new alleles) into USA commercial production.

Materials and Methods

Sources of bees

Worker bees used in this study were collected from single colonies from 18 apiaries representing 15 queen breeders in the USA in 2006, and 24 apiaries from 21 queen breeders in Italy in 1992 (Fig. 1). Queen breeders from the USA, identified by advertisements in beekeeping journals, supplied worker bees representing Italian stock. Two apiaries supplied workers from more than one stock (one colony per stock type). Worker bees were shipped live and stored at -70°C until DNA was extracted.

Bees from Italy were collected from queen breeders in the Emilia-Romagna, Toscana, and Umbria regions. Live bees were placed on ice, then frozen in liquid nitrogen and transported back to the USA where they were stored at -80°C until DNA was extracted. Two bees per colony were sampled to reduce overrepresentation of queen alleles, which would bias the population genetic analyses.

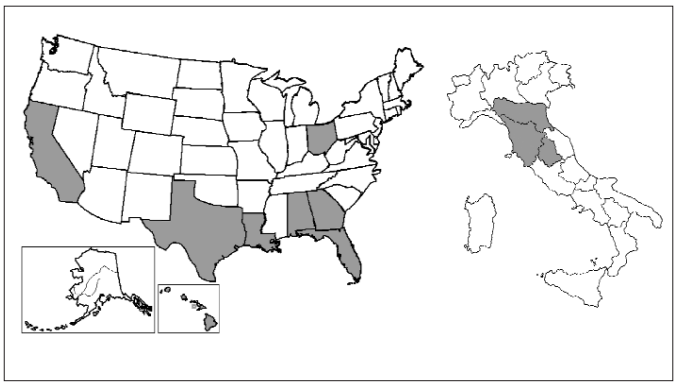

Fig 1. Map showing states of the USA and regions of Italy from which samples of Italian honey bees were obtained for genetic analysis.

DNA extraction

DNA was extracted from the thorax and abdomen (with gut removed) of individual bees. Samples were first homogenized and then treated with Proteinase K (20 mg/ml). 8M Potassium Acetate was then added for protein precipitation, followed by ethanol precipitation and lyophilisation. Pure genomic DNA was rehydrated in Tris/EDTA buffer (0.1M EDTA 8.0, 100mM Tris 8.0) and stored at -20°C.

Genotyping

A total of 14 microsatellite markers (Solignac *et al*., 2003) were screened for polymorphism among the DNA samples. Seven were polymorphic and used for subsequent analyses. Genbank accession numbers and amplification conditions are listed in Table 1. The 5' end of the forward primer was modified by the addition of the complementary sequence to the M13-Cy5-labeled primer (Univ) of sequence GAGTTTTCCCAGTCACGAC. Amplification conditions were optimized on a MJ Research PTC-200 Thermal Cycler®, and were used for all subsequent reactions. The optimized amplification profile was 1 min at 95°C, followed by 40 cycles of 30 sec at 95°C, 30 sec at 52°C or 57°C (see Table 1), and 1 min at 72°C, and ended with 10 min at 72°C. Each 6.5 µl reaction included 1.5 pmol of each primer (MF, R, and Univ); 2 mM of each nucleotide (dNTP); 1X PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 % (w/v) gelatin; Sigma, St. Louis, MO); 0.35 U JumpStart™ Taq polymerase (Sigma); and template DNA (50–100 ng).

Table 1. Locus information for microsatellite loci used for assessment of genetic diversity among Italian honey bees in the USA and Italy. Primer sequences have been previously published (Solignac *et al*., 2003).

Locus	GenBank Acc. No.	Repeat Unit	Ta (°C)	USA Allelic Richness	Gene diversity	Italy Allelic Richness	Gene diversity
Am174	AJ509405	(GGA)10	57	11.883	0.835	8.839	0.779
Am235	AJ509466	(TCC)9	52	4.000	0.363	4.677	0.236
Am306	AJ509537	(GAA)20	52	5.921	0.646	7.585	0.748
Am355	AJ509586	(GAG)11	52	7.782	0.718	6.681	0.719
Am440	AJ509671	(TTTC)5	52	2.921	0.342	5.729	0.752
Am520	AF140070	(TTG)9	57	4.995	0.683	4.960	0.753

Amplification products were analyzed with an ALFexpress Genetic Analyzer (Pharmacia Biotech, Piscataway, NJ) and Fragment Manager v1.2 software (Pharmacia Biotech). Six µl of PCR product were mixed with size standards and loading buffer (100 mM bromophenol blue in deionised formamide). The mixture was heated for 4 min at 95°C and loaded onto 6% Page Plus (Amresco, Inc., Solon, OH) polyacrylamide gels containing 6 M urea. The products were separated at 1500 V, 60 mA, 25 W, 55°C with a sampling interval of 1 sec.

Data Analysis

The software MICROCHECKER (van Oosterhout *et al*., 2004) was used to assess the presence of null alleles at each locus within each population. Measures of allelic variation (allelic richness (mean number of alleles per locus), gene diversity (heterozygosity), and F_{IS} inbreeding coefficient)) were generated in FSTAT (Goudet, 2001) using resampling to account for differences in sample size. Assessment of population genetic structure was completed using Weir and Cockerham's (1984) estimators. Additionally, global and pairwise values of θ (Wright's (1951) F_{ST}) were estimated in FSTAT. F_{ST} estimates the amount of genetic diversity that can be attributed to between population differences compared to within population diversity. The values range from 0 to 1, with 0 representing homogeneous populations and 1 representing complete isolation and differentiation between populations. Values of F_{ST} are typically lower for microsatellite loci than other genetic markers, as F_{ST} is limited by heterozygosity. Standard errors were calculated by jackknifing over loci. Significance was determined with tests based on 10,000 permutations generated in FSTAT. Genetic distance, calculated as chord distance (Cavalli-Sforza and Edwards, 1967), was calculated in Genetix (Belkhir *et al*., 1996) and neighbour-joining phenograms were generated from chord distance data in MEGA3.1 (Kumar *et al*., 2004). The software BOTTLENECK (Cornuet and Luikart, 1996) was used to test for recent reductions in effective population size. In this test, gene diversity excess was estimated using the Wilcoxon signed rank test and the mode-shift of allele frequency was assessed. The two-phase model (TPM) and the stepwise mutation model (SMM) were used. For the TPM test, both the number of multistep changes and the variance were set at 10%. All statistics for BOTTLENECK were calculated using 10,000 iterations.

Results

A total of 14 microsatellite loci were screened for polymorphism between USA and Italian sources of Italian bees. Ten were polymorphic and seven demonstrated consistent amplification. Of these, six were used for further analyses (Table 1). The locus (Am504) had null alleles present and was removed. A total of 48 alleles at the six remaining loci were identified from all USA and Italian samples. The USA (n = 19) and Italian (n = 24) samples each had a total of 38 alleles. The alleles for each population overlapped, but were not identical. Both USA and Italian populations had 10 unique alleles. Allelic richness differed slightly between USA and Italian populations but was highly varied among loci (Table 1). Gene diversity also varied among loci, but was similar between USA and Italian bees for each locus. F_{IS} estimates were low for both USA and Italy (-0.063 and 0.135, respectively) and did not differ ($P > 0.05$). There was no evidence for recent bottlenecks amongst either the USA or Italian apiaries based on patterns of allele frequencies ($p > 0.05$ for SMM and TPM) and mode shift of allele frequencies.

Apiaries within the USA were divided regionally into "California" and "Rest of the USA" following Schiff and Sheppard's (1995) designation. Regional differentiation was evident with respect to F_{ST} estimates ($F_{ST} = 0.0058$; $P = 0.0087$). In Italy, 2 apiaries in Toscana and Umbria and 22 in the Emilia-Romagne were sampled. Because of unequal sample size, only the samples from Emilia-Romagne were used in F_{ST} analysis. Comparisons between USA and Italy showed differentiation by region in the USA and globally ($F_{ST} = 0.1675$; $P = 0.0500$).

Chord distances (Cavalli-Sforza and Edwards, 1967) were used to generate a neighbour-joining phenogram for USA apiaries using Italian samples as an outgroup (Fig. 3). Some clustering of samples from California was evident, but division among regions was not complete.

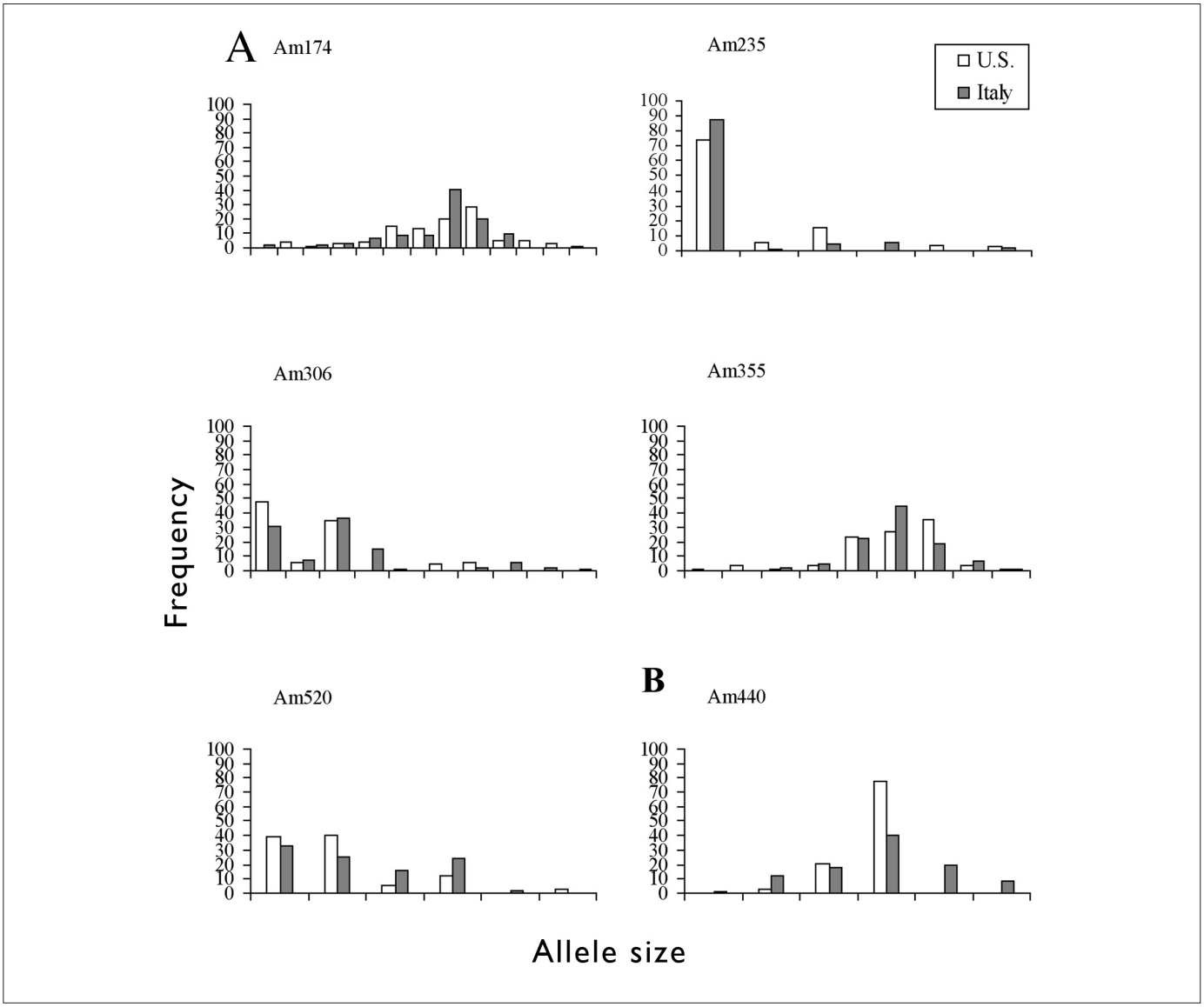

Fig 2. Microsatellite allele frequencies at 6 loci used in genetic diversity analyses of Italian honey bees in the USA and Italy. Loci are grouped according to repeat unit length as follows: A) trinucleotide repeats, B) tetranucleotide repeat.

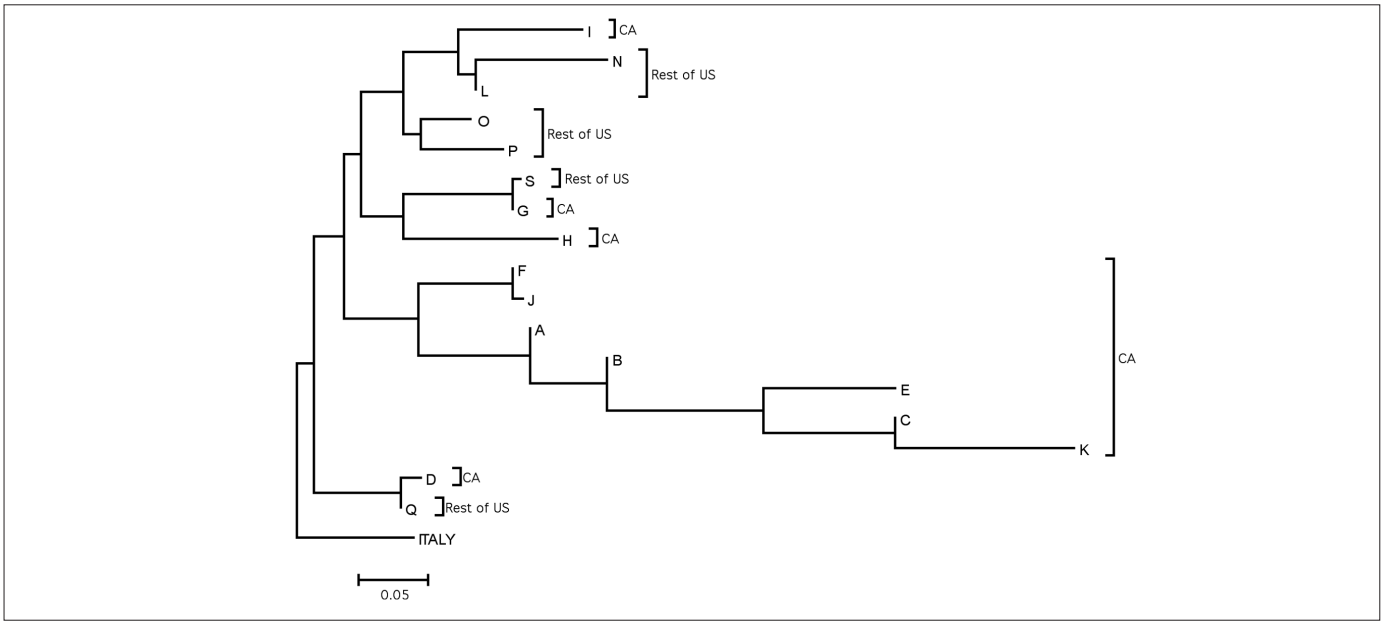

Fig 3. Neighbour-joining phenogram of Italian bees in the USA and Italy. The phenogram was generated from chord distances (Cavalli-Sforza and Edwards, 1967) based on genotypes of 6 microsatellite loci. Letters represent queen breeders from which samples were collected.

Discussion

Microsatellite variation was evident amongst honey bees from all sources. Allelic richness and gene diversity varied among loci in both the USA and Italy. Allelic richness was slightly higher in the USA whereas gene diversity was very similar between the two countries. While these two parameters are related, it has been suggested that allelic richness is a better reflection of a population's long-term evolutionary potential than gene diversity, because gene diversity reflects the short-term potential for recovery from a population bottleneck (Allendorf, 1986; Petit *et al*., 1998). This is, in part, because allelic richness is more sensitive to severe bottlenecks (Nei *et al*., 1975; Leberg, 1992; Spencer *et al*., 2000). A reduction in the number of alleles would have a more significant evolutionary impact than would a reduction in heterozygosity that could be quickly reversed by the introduction of new alleles through gene flow.

Values for both measures were relatively high for USA and Italian samples indicating that long-term persistence of both populations and recovery from a population bottleneck would be likely. In heavily fragmented populations, with little or no gene flow between them, we would expect to see much lower levels of allelic richness and significant departure from HWE as was the case for island populations of the bumble bee, *Bombus sylvarum*, (Ellis *et al*., 2006) and the Balearic honey bee, *A. mellifera* (De La Rua *et al*., 2003; Ellis *et al*., 2006). A similar trend was evident for allozyme polymorphisms in a comparison of specialist versus generalist bees in Chile where allelic richness was reduced in the more heavily fragmented specialist bees (Packer *et al*., 2005).

Low F_{IS} (inbreeding coefficient; Weir and Cockerham, 1984) values were measured in both populations, indicating no short-term potential for inbreeding. There are multiple factors inherent in beekeeping practices that promote allelic diversity, hence reducing the risk of inbreeding. Breeding practices focus largely on performance, which may include multiple familial (genetic) lines. The queen breeding component of the industry also contributes to allelic diversity, in part, because queens are regularly sold by queen breeders and subsequently transported to beekeepers throughout each country. In this respect, current beekeeping practices promote diversity and do not necessarily provide an opportunity for local differentiation to occur. When a new queen is introduced into an apiary, her alleles are then perpetuated through drones she produces which will in turn contribute to the genetic variation of subsequent generations in that region upon mating. Because the sources of queens are limited, this should effectively homogenize populations (apiaries) and maintain diversity across regions, as was suggested by Schiff and Sheppard (1995).

Despite the artificial "migration" (i.e., gene flow) facilitated by the beekeeping industry, estimates of population-level differentiation (F_{ST}) revealed significant differences among apiaries within the USA when grouped by region. While F_{ST} estimates were significant within the USA, the values were low. However differentiation between California apiaries and those in the remaining portions of the USA was consistent with patterns of population structure reported for allozymes (Schiff and Sheppard, 1995; 1996). Comparisons of genetic distance data with a neighbour-joining phenogram showed some clustering within the two subsets of USA bees (California vs. the rest of USA). This clustering was not related to subspecies or strain of bee tested. This differentiation could, in part, be due to regional preference for stock sources.

As was expected, comparisons of Italian bees in the USA and Italy revealed more pronounced differentiation. This probably reflects breeding efforts and geographic isolation for the past 85 years. Another important consideration is that "Italian" bees in the USA are actually a mixture of subspecies of *A. mellifera* due to various introductions into the USA. This, in effect, introduces new alleles into the population which may contribute to differentiation and elevated levels of allelic richness.

One potential issue with the comparison of microsatellite allele frequencies across such a large geographic scale (USA to Italy) is the high likelihood of size homoplasy of alleles (i.e., different alleles of the same size for a particular locus arising from different origins in geographically and genetically isolated populations) due to the high mutation rate associated with microsatellite loci (Estoup *et al*., 1995). This could bias differentiation levels downward. However the time of isolation on an evolutionary time scale is relatively short. While considering the potential homogenizing affects of size homoplasy, the data presented here are still significant and differentiation was still evident.

Tests for recent bottlenecks under both the SMM and the TPM were negative for both populations despite the pressure initial that infestations with *Varroa destructor* placed on populations in both countries. One possible explanation for the lack of bottlenecks is that beekeepers have generally taken measures to control *V. destructor* and thus avoided bottlenecks. However bottlenecks have been demonstrated in managed honey bee populations in the Balearic Islands in the Mediterranean where *V. destructor* infestations coupled with introductions of small numbers of colonies have occurred (De La Rua *et al*., 2003).

The data presented here suggest that genetic diversity among commercial beekeeping operations in both the USA and Italy is relatively high and that inbreeding depression is not an immediate concern. Recent declines in honey bee populations in the USA have been well-documented (Cox-Foster *et al*., 2007; Van Engelsdorp *et al*., 2007). While the cause of these declines is unknown, this only emphasizes the need for maintenance of high levels of genetic diversity in bee populations to avoid the potential for catastrophic population decline from pests and diseases.

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