J Insect Conserv (2008) 12:527–537
DOI 10.1007/s10841-007-9093-6

ORIGINAL PAPER

Morphological and molecular characterization of the Landes honey bee (*Apis mellifera* L.) ecotype for genetic conservation

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Received: 13 March 2007 / Accepted: 21 May 2007 / Published online: 6 July 2007

Abstract A population of honey bees (*Apis mellifera mellifera* L.) with an annual colony brood cycle adapted to a locally abundant floral source in the Landes region of Southwest France is the subject of genetic conservation efforts. This population is maintained by local beekeepers in an area that experiences both an annual seasonal influx of non-local colonies and the permanent culture of imported stock. However, some colonies native to the Landes do not have the adapted brood cycle and their status as ecotypic are in question. The present study used morphology, mitochondrial DNA and microsatellites to characterize the endemic population and suggests further genetic conservation strategies. These methods yielded different degrees of discrimination of native and imported colonies and provided a powerful suite of tools for local resource managers. Colonies from the Landes could be differentiated from non-local French *A. m. mellifera* populations using morphometric analysis, and from non-native and reference populations using mtDNA and microsatellites. Seven morphological characters were identified by discriminant analysis as informative for delineating the Landes ecotype from other *A. m. mellifera* populations. Mitochondrial haplotypes for the population were characterized and five microsatellite loci were found to be informative in characterizing the Landes population. Asymmetric gene flow detected with microsatellite alleles was observed to be 5.5–5.9% from imported to native stocks of honey bees while introgression of native microsatellite alleles into imported colonies was 21.6%.

Keywords *Apis mellifera* · Ecotype conservation · Microsatellites · Mitochondrial DNA · Morphometrics

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Introduction

The need to conserve the genetic diversity of domesticated plants is well documented (Rogers 2004 and references therein). More recently, Scherf (2000) drew attention to the loss of genetic diversity in livestock world wide, focusing on domesticated mammals and birds. Despite the widespread consensus that preserving the genetic diversity of domesticated species may prove valuable to humanity, there have been few efforts to preserve the genetic diversity of beneficial arthropod species. While several arthropod species are cultured by humans, *Apis mellifera* L., the western honey bee, is the most economically important beneficial insect (Delaplane and Mayer 2000). The contribution of honey bees to crop pollination is estimated to be tens of billions of US dollars in value to crops worldwide (Delaplane and Mayer 2000; Carreck and Williams 1998; Borneck and Merle 1989).

The honey bee has proven to be highly adaptive to a wide variety of ecosystems in its native range of Africa, Europe, and central and western Asia. About 26 subspecies

and numerous "ecotypes" of *A. mellifera* have been described based upon behavior, morphology, and molecular evidence (Ruttner 1988; Sheppard et al. 1997; Sheppard and Meixner 2003). While several subspecies inhabit large geographic areas, some subspecies (and all of the ecotypes) inhabit relatively small geographic regions with smaller population sizes. The risk to the genetic integrity of small populations is underscored by the fact that human mediated movement of honey bee stock has produced significant changes in honey bee population genetic structure. Notably, endemic populations of *A. m. mellifera* in Germany were almost completely replaced with imported *A. m. carnica* in an effort to improve managed bee stocks beginning in the 1930s (Ruttner 1988; Kauhausen-Keller and Keller 1994). More recently, the massive hybridization of European and African stocks in tropical and sub-tropical America (Schneider et al. 2004) attests to the possibility of large scale genetic change. While large scale introgression events are dramatic, smaller events may be more insidious in that genetic diversity can be lost rapidly as native populations are killed by introduced parasitic mites or replaced by imported stock. The possibility of such genetic introgression has been noted for Malta (Sheppard et al. 1997), the Canary Islands (De La Rúa et al. 2001, 2002) and the Balearic Islands (De La Rúa et al. 2003) among others.

Following the introduction and establishment of the invasive parasitic mite *Varroa destructor* Anderson and Truman into the range of *A. mellifera*, the task of conserving small endemic populations of honey bees as potential breeding stock for future generations became more difficult and increasingly urgent (Moritz et al. 2007). De La Rúa et al. (2003) observed a genetic bottleneck on a Balearic Island (Spain) apparently due to a recent massive loss of endemic colonies to *V. destructor* infestation. Additionally, the same study found significant genetic introgression from imported stock on nearby islands presumed to result from beekeepers importing queen bees (De La Rúa et al. 2003). While island populations are at high risk of extinction and introgression events, small endemic populations throughout the native range of *A. mellifera* are subject to similar pressure through migratory beekeeping and *V. destructor* infestation.

Within France, Louveaux et al. (1966) described four ecotypes of the subspecies *Apis mellifera mellifera*. The ecotypes were distinguished from one another by distinct colony population cycles (as measured by the annual brood cycle) that were genetically adapted to the floral phenology within their areas of endemism. However, in the 40 years since the description of these ecotypes, transhumance of honey bee colonies (Odoux and Garnery 1995) and the permanent culture of imported bee stocks (Perrier et al. 2003) have increased within France.

One of the four French ecotypes occurred in the Landes region and had an unusual colony annual brood cycle that was adapted to the late season blooming of ling heather, *Calluna vulgaris* L. (Louveaux 1973). Cornuet et al. (1982) conducted a study of a limited suite of morphological traits of the Landes ecotype and found low levels of introgression from introduced races and described two characters that were useful to differentiate the ecotype from other French honey bee populations. Specifically, they found that the length of the yellow band on the second abdominal tergum and the length of hair on the on the fifth abdominal tergum distinguished the Landes ecotype from other French *A. m. mellifera* populations.

Perrier et al. (2003) found low levels of mtDNA and microsatellite introgression from imported stock into the Landes population, despite the local year-round culture of imported stock and seasonal movement of colonies into the region by beekeepers to exploit the heather honey crop. Specifically, the authors reported that 27.8% of the colonies sampled within the Landes region had C lineage mitochondrial DNA characteristic of eastern European honey bees and the imported Buckfast hybrid and 24.03% of the colonies were assigned as introduced colonies using multilocus microsatellite genotypes (*ibid*). Despite this apparent high level of genetic introgression, they observed significant population substructure when viewed at the apiary level and a low level of nuclear introgression (3.09%) from imported Buckfast honey bees into the local *A. m. mellifera*. Thus, a large proportion of the honey bee colonies in the Landes were non-native in origin, but individual beekeepers were maintaining relatively pure stock nonetheless.

Strange et al. (2007) demonstrated the persistence of the Landes ecotype, through a reevaluation of the colony annual brood cycle. The annual brood cycle described by Louveaux et al. (1966) was found in nearly 50% of the colonies surveyed by Strange et al. (2007), yet results were complicated by the fact that a large proportion (>90%) of the Landes colonies classified as non-ecotypic had a reproductive swarming event during the experiment. One solution would be to re-evaluate the brood cycle in those colonies for another year; however, annual brood cycle measurements are difficult and time consuming and present a major hurdle for resource managers who wish to establish an in situ genetic conservatory in the Landes. Molecular and morphological characters associated with the Landes ecotype would be useful to screen large numbers of candidate colonies targeted for conservation prior to the annual brood cycle analysis. The ideal situation would be to find several morphological or molecular markers that are highly correlated with the ecotype, thus reducing the need for the difficult evaluation of colony population cycle.

Here we present a combined analysis of morphology, mtDNA and microsatellite data to provide a quantitative means of selecting the Landes ecotype targeted for conservation. The present study focuses on the description of colonies known to have the Landes ecotypic brood cycle trait whereas previous studies sampled colonies with no known brood cycle data to inform them (Cornuet et al. 1982; Perrier et al. 2003). We also discuss the potential for applying this methodology more widely to genetic conservation projects.

Materials and methods

Honey bee samples

Samples of worker bees were collected into 70% ethanol from the brood area of 29 colonies of *A. m. mellifera* located in the Landes region of Southwest France in the fall of 2002. The annual brood cycle behavior of these colonies had been evaluated the preceding spring and summer; 14 were characterized as having the ecotypic annual brood cycle, while the remaining 15 colonies were designated as non-ecotypic for the present study. This latter group may have included ecotypic colonies that had been scored as non-ecotypic colonies due to interruption in their brood cycles (Strange et al. 2007). Samples of workers were also taken from the brood area of 41 non-native colonies of Buckfast hybrid bees (a commercially available stock developed from crosses of several subspecies) located in an apiary 2.1 km from one of the *A. m. mellifera* apiaries.

Morphometric analysis

About 15 workers from each Landes *A. m. mellifera* colony were measured for a suite of 36 morphologically informative characters following Ruttner et al. (1978). Wing venation and size characters were measured with a microscope mounted CCD camera and Bee2© software (Meixner and Meixner 2004). Pilosity and pigmentation characters were measured using a microscope and ocular micrometer. A discriminant analysis with reference populations of five subspecies (*A. m. carnica* (n = 111), *A. m. caucasica* (n = 27), *A. m. ligustica* (n = 38), *A. m. mellifera* (n = 62) and *A. m. iberica* (n = 4)), where n is the number of colonies, was performed to determine if the Landes honey bees were differentiated from other populations of the *A. m. mellifera* subspecies. A second discriminant analysis was performed with only the *A. m. mellifera* and *A. m. iberica* (from Spain) reference samples to identify the informative characters useful for the discrimination of the Landes honey bees from other populations of the *A. m. mellifera* subspecies. Characters extracted by the discriminate analysis were compared using an independent samples t-test between ecotypic and non-ecotypic Landes colonies. Population statistics, principal component analysis, discriminant analysis and t-tests were performed using SPSS v12.1 (2003). Reference populations of *A. m. carnica*, *A. m. caucasica*, *A. m. ligustica*, *A. m. mellifera*, and *A. m. iberica* were obtained from the database of the Institut für Bienenkunde in Frankfurt, Germany.

Molecular analysis

DNA extraction

In addition to the 29 native Landes colonies used in the morphometric analysis, workers were collected from a reference apiary in the Landes region, comprised of 41 colonies of the imported hybrid Buckfast stock. A reference population of non-ecotypic *A. m. mellifera* from Ille-sur-Tet, France (300 km east of the Landes) was included in the analysis. DNA was extracted from the metathoracic leg of 24 individual workers from each colony of the ecotypic (n = 14), non-ecotypic (n = 15), and Buckfast (n = 41) Landes populations in 150 µl 10% Chelex and 5 µl proteinase K (10 mg/ml) extraction solution (modified from Walsh et al. 1991), incubated in a thermocycler for 1 h at 55°C, 15 min at 99°C, 1 min at 37°C and 15 min at 99°C. DNA was extracted from one worker from each of the Ille-sur-Tet reference colonies (n = 18) according to the same protocol. Extracted DNA was stored at –80°C until used for amplifications.

MtDNA analysis

The $tRNA^{leu}$-COII intergenic region was amplified from four worker bees from each colony using the E2 and H2 primer pair according to Cornuet et al. (1991). Four bees from each colony were chosen to eliminate the possibility of incorrectly basing the colony haplotype on a worker that had "drifted" into the colony from a neighboring hive. The resulting amplified DNA fragments were electorphoretically separated on 1.4% agarose gels. Length polymorphisms were scored for each bee in increasing length as Q, PQ, PQQ, or PQQQ (200, 250, 450, and 650 base pairs, respectively) following Garnery et al. (1993). A 20 µl aliquot of each amplified sample was digested at 37°C for 6 h with five units of the DraI restriction enzyme (Promega, Madison, WI) and visualized on a 10% acrylamide gel. Resulting mitochondrial haplotypes were scored based upon restriction fragment lengths according to Garnery et al. (1993, 1998a). The conventional honey bee

haplotype names were used, where A, C, and M refer to the African, west European, and north Mediterranean mitochondrial lineages, respectively. An exact test of sample differentiation based on haplotype frequencies was calculated according to Raymond and Rousset (1995) using Arlequin population genetic software (Schneider et al. 2000) for the ecotypic, non-ecotypic and reference (Ille-sur-Tet and Buckfast) populations.

Microsatellite analysis

About 10 polymorphic microsatellite loci (A7, A24, A28, A88, A113, B124 (Estoup et al. 1995), Ap43, (Garnery et al. 1998b) Ap55, Ap66, and Ap81 (Solignac et al. 2003)) amplified in two multiplex reactions were analyzed. Extracted DNA was amplified by PCR in 10 µl reactions containing 1 µl extracted DNA, 1 × Promega (Madison, WI) reaction buffer, 3 mM dNTP mixture, 1.0–4.0 mM primer, 0.001 mg bovine serum albumin, 1.5 units Taq polymerase (Promega, Madison WI) and the $MgCl_2$ concentration was adjusted to 1.5 mM for loci A24, A28, A88, Ap66, and B124 and 1.2 mM for loci A7, A113, Ap43, Ap55, and Ap81. The PCR conditions for all reactions were one 7 min cycle at 95°C, 30 cycles of 95°C for 30 s, 54°C for 30 s, 72°C for 30 s and a 60 min cycle at 72°C. The amplifications were performed with florescent dye-labeled primers and separated on an Applied Biosystems 3730 automatic sequencer. The resulting electrophoretograms were scored using GeneMapper™ Software (Applied Biosystems).

Queen genotypes for the Landes colonies (Buckfast, ecotypic, and non-ecotypic colonies) were inferred from the 24 worker genotypes. Population allele frequencies were calculated using queen genotypes for the Landes samples and from one worker per colony for the Ille-sur-Tet samples. Tests for Hardy-Weinberg equilibrium and population pairwise Fst scores were calculated using Arlequin population genetics software (Schneider et al. 2000). To select informative genes for population screening, a locus by locus AMOVA (Weir and Cockerham 1984) was performed using the 10 loci using Arlequin software (Schneider et al. 2000). Analysis of the population structure and estimation of nuclear introgression was performed with STRUCTURE software (Prichard et al. 2000) using only the queen genotypes from the ecotypic, non-ecotypic and Buckfast queens located in the Landes.

Results

Morphometric analysis

A principal component analysis of the ecotypic and non-ecotypic Landes colonies placed all Landes colonies into a distinct cluster apart from all reference populations, including *A. m. mellifera* from several locations in Europe and *A. m. iberica* from Spain. This separation was confirmed by discriminant analysis. A second discriminant analysis failed to separate Landes colonies with the ecotypic brood pattern from those that did not have the ecotypic brood cycle; however all Landes colonies clearly separated from *A. m. mellifera* and *A. m. iberica*. Figure 1 plots discriminant factor 1 (accounting for 93.0% of the variation) against discriminant factor 2 (accounting for 6.7% of the variation) for the ecotypic and non-ecotypic Landes colonies and *A. m. mellifera* and *A. m. iberica*. All Landes colonies clustered together in both discriminant analyses, making differentiation of ecotypic versus non-ecotypic Landes colonies difficult using morphological characters.

The discriminate analysis of ecotypic and non-ecotypic Landes colonies, *A. m. mellifera* and *A. m. iberica* reference colonies revealed that seven morphological characters from the suite of 36 sampled characters were most informative for the analysis. Table 1 shows the mean ± standard deviation for each of the characters for the ecotypic colonies, non-ecotypic colonies, *A. m. mellifera* and *A. m. iberica* reference samples. Informative characters identified by the discriminant analysis were compared between the ecotypic and non-ecotypic Landes colonies. Significantly different means were found between the two groups for two of the characters in the analysis: width of tomentum dark stripe (t = –2.65; df = 26; P = 0.014) and

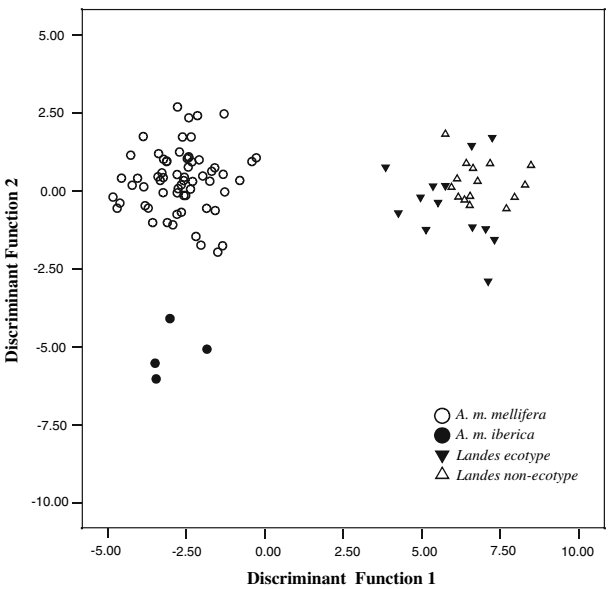

Fig. 1 Discriminate analysis of morphological data. Samples include ecotype and non-ecotype Landes populations and the *A. m. mellifera* and *A. m. iberica* reference samples. Discriminant function 1 accounts for 93.0% of the variation and discriminant function 2 accounts for 6.7% of the variation among populations

Table 1 Values are means and standard deviations of samples

Character	Landes ecotype (n = 14)	Landes non-ecotype (n = 15)	*A. m. mellifera* (n = 62)	*A. m. iberica* (n = 4)	Cornuet et al. (1982) (n = 84)
Length of hair on tergum 5	39.67 ± 3.41	40.79 ± 4.37	37.19 ± 5.76	30.39 ± 1.64	40.20 ± 11.9
Length of proboscis	628.87 ± 11.35	640.11 ± 16.10	611.62 ± 12.92	631.75 ± 1.80	624.7 ± 6.8
Width of tomentum dark stripe*	62.96 ± 10.94	60.25 ± 9.15	55.42 ± 7.96	57.42 ± 37.22	
Width of tomentum light stripe*	81.50 ± 7.71	88.99 ± 7.27	89.35 ± 12.81	85.41 ± 3.11	78.70 ± 3.6
Pigment of tergum 3*	3.99 ± 0.34	3.80 ± 0.37	3.28 ± 0.94	1.45 ± 0.46	
Length of tergum 3*	219.53 ± 7.15	221.31 ± 5.48	234.23 ± 4.68	234.61 ± 5.22	
Length of forewing*	920.56 ± 12.21	920.28 ± 6.39	928.10 ± 17.45	963.85 ± 7.40	
Wing vein angle g18*	99.66 ± 1.13	100.76 ± 1.37	97.10 ± 2.34	94.97 ± 0.67	
Wing vein angle j16*	94.02 ± 3.06	94.03 ± 1.71	97.27 ± 1.77	100.53 ± 0.67	
Cubital index	1.77 ± 0.11	1.83 ± 0.20	1.80 ± 0.18	1.65 ± 0.04	1.797 ± 0.13

Samples include colonies from the Landes region that exhibited the characteristic Landes ecotypic brood cycle, colonies from the Landes that did not exhibit the ecotypic brood cycle, samples from two European subspecies, *A. m. mellifera* and *A. m. iberica*, and samples from a previous study of Landes honey bees by Cornuet et al. (1982). Each sample represents a colony and n is the number of colonies used in the analysis. Measurements of sizes are in units of 1/100 mm and wing vein angles are degrees. Measurements made according to Ruttner (1988). Asterisks (*) indicate characters extracted by discriminant analysis.

wing vein angle g18 (t = –2.44; df = 27; P = 0.022). The means and standard deviations of the characters are given in Table 1 together with the comparable data available from Cornuet et al. (1982).

Mitochondrial DNA

Table 2 shows the frequencies of mtDNA haplotypes produced by the DraI restriction digestion of the $tRNA^{leu}$-COII intergenic region. The most common haplotype of ecotypic colonies was M4 (71.4%), followed by M4' (14.3%) and M17 (14.3%). The non-ecotypic Landes population was also primarily composed of the M4 haplotype (80.0%) with M4', M19 and C1 mitochondrial haplotypes each represented by 6.7% of the population. The exact test of sample differentiation based upon haplotype frequencies showed no significant differentiation of Landes ecotypic colonies and non-ecotypic colonies (P = 0.487 ± 0.009), although both were significantly different from the Buckfast and Ille-sur-Tet reference populations at the P = 0.05 level.

Microsatellite analysis

Pairwise Fst comparisons for the four groups showed no significant differences in microsatellite allele frequencies between the Landes ecotypic and non-ecotypic colonies at the P = 0.05 significance level (P = 0.486 ± 0.045). The Ille-sur-Tet and Buckfast populations were significantly different from each other and the Landes (ecotypic and non-ecotypic) populations at the P = 0.05 level. Allele frequencies at each locus, number of alleles per locus, expected and observed heteroygosity and percent of polymorphic loci for each population are given in Table 3. Of the 10 microsatellite loci screened, five explained more than 30.0% of the among population variation between Landes colonies and the imported Buckfast population as detected with the locus by locus AMOVA. The five loci were A88, A24, A28, Ap66, and Ap81.

Table 2 Relative mitochondrial haplotype frequencies for Landes populations and reference populations where n is the number of colonies sampled

Haplotype	Ecotype (n = 14)	Non-ecotype (n = 15)	Buckfast (n = 41)	Ille-sur-tet (n = 15)
M4	0.714	0.800	0	0.267
M4'	0.143	0.067	0	0.133
M17	0.143	0	0	0
M19	0	0.067	0	0.333
M31	0	0	0	0.267
C1	0	0.067	0.780	0
C2	0	0	0.024	0
C3	0	0	0.171	0
A1	0	0	0.024	0
D	0.484 ± 0.143	0.371 ± 0.153	0.369 ± 0.084	0.781 ± 0.053

Unbiased haplotype diversity (D) and standard deviation is given for each population

Table 3 Microsatellite allele frequencies (locus × population) at 10 loci for four honey bee populations where *n* is the number of alleles sampled

Locus	Allele (bp)	Ecotype (*n* = 28)	Non-ecotype (*n* = 30)	Buckfast (*n* = 82)	Ille-sur-Tet (*n* = 36)
A7	105				0.028
	107	0.036		0.037	0.139
	109				0.111
	111	0.821	0.867	0.354	0.667
	113	0.107	0.033	0.024	
	116*		0.033	0.305	0.028
	118			0.012	
	120*		0.033		
	122			0.012	
	128			0.012	0.028
	130			0.049	
	132			0.073	
	134	0.036			
	136		0.033		
	140			0.024	
	142			0.024	
	154			0.012	
	160			0.061	
	Hd	0.381	0.253	0.776	0.537
	Hp	0.357	0.267	0.829	0.667
A24	98	0.964	0.967	0.415	0.944
	100				0.028
	104			0.024	
	106	0.036	0.033	0.281	0.028
	108*			0.281	
	Hd	0.14	0.131	0.688	0.162
	Hp	0.071	0.067	0.683	0.111
A28	132	0.786	0.833	0.243	0.889
	134*			0.049	
	138*	0.107	0.067	0.707	0.028
	144	0.107	0.1		0.083
	Hd	0.373	0.356	0.46	0.257
	Hp	0.429	0.267	0.537	0.111
A88	143		0.067	0.207	
	146	0.964	0.933	0.207	0.972
	152*	0.037		0.293	0.028
	154*			0.268	
	Hd	0.14	0.191	0.755	0.11
	Hp	0.071	0.133	0.78	0.056
A113	202	0.071	0.067	0.024	0.028
	214*	0.036	0.067	0.207	
	220	0.821	0.767	0.634	0.917
	222	0.036	0.033	0.024	0.028
	224			0.012	0.028

Table 3 continued

Locus	Allele (bp)	Ecotype (n = 28)	Non-ecotype (n = 30)	Buckfast (n = 82)	Ille-sur-Tet (n = 36)
	226		0.067	0.061	
	228			0.037	
	236	0.036			
	Hd	0.386	0.411	0.555	0.213
	Hp	0.286	0.467	0.634	0.111
B124	214			0.024	
	216			0.195	
	218			0.061	
	220	0.071	0.167	0.11	
	222	0.286	0.167	0.134	0.194
	224	0.143	0.267	0.061	0.25
	226	0.107	0.1	0.024	0.333
	228		0.067	0.024	
	230		0.033	0.012	
	232	0.143	0.033	0.037	0.111
	234		0.033	0.11	0.028
	236	0.036	0.067	0.024	
	238	0.179	0.067	0.012	
	240				0.028
	242	0.036		0.171	0.028
	244				
	246				0.028
	Hd	0.857	0.876	0.89	0.825
	Hp	1	1	0.951	0.722
Ap66	95			0.622	0.029
	100	0.786	0.8	0.354	0.088
	101	0.214	0.2		0.618
	103				0.265
	113			0.024	
	Hd	0.349	0.384	0.509	0.556
	Hp	0.429	0.4	0.537	0.706
Ap43	135	0.393	0.433	0.073	0.25
	137	0.571	0.567	0.5	0.679
	139			0.012	
	143*			0.122	
	145*			0.134	
	147*			0.11	0.071
	149			0.012	
	151			0.012	
	161			0.012	
	167			0.012	
	181	0.036			
	Hd	0.537	0.508	0.708	0.489
	Hp	0.857	0.6	0.659	0.357

Table 3 continued

Locus	Allele (bp)	Ecotype (n = 28)	Non-ecotype (n = 30)	Buckfast (n = 82)	Ille-sur-Tet (n = 36)
Ap81	128	0.964	0.933	0.268	0.611
	130				0.028
	136	0.036	0.033	0.732	
	146				0.028
	148		0.033		0.333
	Hd	0.071	0.193	0.433	0.579
	Hp	0.14	0.133	0.463	0.444
Ap55	171			0.049	
	173		0.033	0.232	0.028
	175	0.107	0.033	0.366	0.028
	177	0.143	0.167	0.061	0.056
	179	0.571	0.5	0.159	0.361
	181	0.179	0.2	0.11	0.25
	183		0.067	0.012	0.278
	200			0.012	
	Hd	0.677	0.699	0.778	0.759
	Hp	0.5	0.867	0.854	0.611

	Ecotype	Landes	Buckfast	Ille-sur-Tet
No. alleles per locus	3.5 ± 1.9	4.0 ± 2.6	6.4 ± 3.9	4.1 ± 1.8
Polymorphic loci (%)	100	100	100	100
Hp averaged	0.398 ± 0.075	0.400 ± 0.238	0.655 ± 0.049	0.449 ± 0.079
Hd averaged	0.407 ± 0.320	0.420 ± 0.317	0.689 ± 0.154	0.377 ± 0.269

Expected (Hd) and observed (Hp) heterozygosity are given for each locus. Average number of alleles per locus, percent polymorphic loci, average Hd and Hp, with standard deviations are given for each population. Asterisks (*) indicate putative C lineage diagnostic alleles (Garnery et al. 1998b).

Assignment of individual colonies allowed for estimation of the number of populations (K) (Prichard et al. 2000) occurring in the Landes region. The posterior probability that there were three inferred populations (K = 3) in the Landes experimental colonies (ecotypic, non-ecotypic, and Buckfast) was greater than the probability for other values of K. However, differences in the ln Pr(x|K) were less than 50 for K = {2 ... 5} in which case Prichard et al. (2000) advise choosing the smaller value of K. Inferred cluster compositions are given for the K = 2 and K = 3 models in Figs. 2 and 3 respectively. Under the K = 3 model (Fig. 3) a single cluster (Cluster 2) contained 86.2% of the ecotypic and non-ecotypic colonies and one Buckfast colony. The two other clusters were composed primarily of Buckfast colonies, where Cluster 3 was contained only colonies of Buckfast origin and Cluster 1 included two non-ecotypic Les Landes colonies. One ecotypic, one non-ecotypic and three Buckfast colonies occurred in positions intermediate to the three clusters (less than 75% probability of assign-

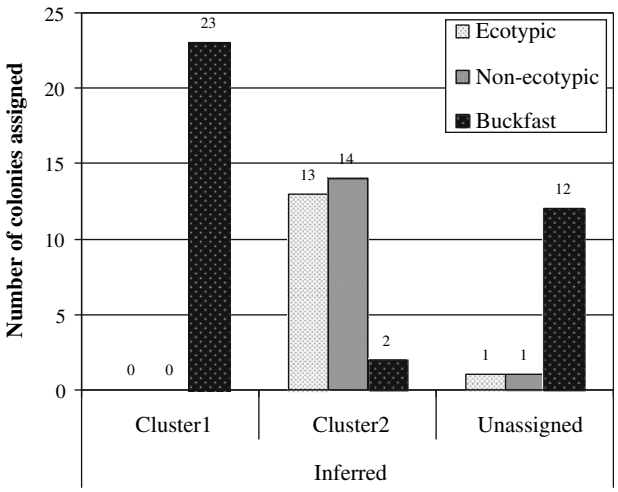

Fig. 2 Number of colonies assigned to clusters of inferred ancestry by initial grouping as ecotypic, non-ecotypic, and Buckfast under the model K (number of populations) is two. Unassigned colonies could not be placed in a cluster with >75% probability

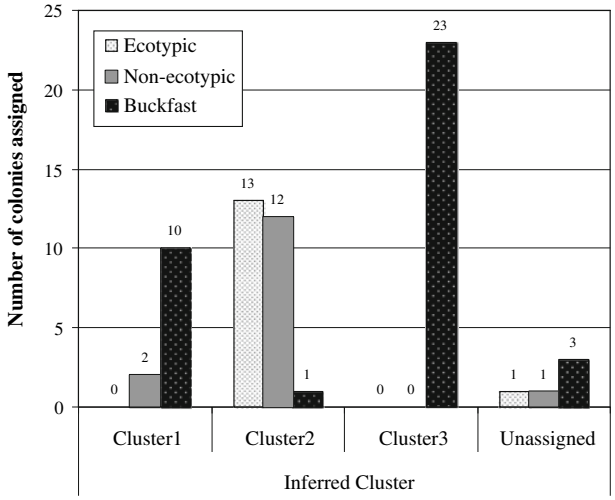

Fig. 3 Number of colonies assigned to clusters of inferred ancestry by initial grouping as ecotypic, non-ecotypic, and Buckfast under the model K (number of populations) is three. Unassigned colonies could not be placed in a cluster with >75% probability

Table 4 Means and standard deviation of estimates of microsatellite allele introgression into three groups of colonies based on assignment of colonies to clusters of inferred ancestry for two models of population structure, where K is equal to the number of populations assumed under the model the model

	% Introgression ± sd
$K = 2$	
Ecotype	5.83 ± 2.97
Non-ecotype	8.17 ± 4.12
Buckfast	21.33 ± 1.86
$K = 3$	
Ecotype	8.03 ± 2.20
Non-ecotype	15.83 ± 2.45
Buckfast	8.10 ± 2.21

ment to a population cluster). Under the $K = 2$ model (Fig. 2), Cluster 1 is composed of 23 Buckfast colonies whereas Cluster 2 includes 13 ecotypic, 14 non-ecotypic, and two Buckfast colonies. One ecotypic, one non-ecotypic, and 12 Buckfast colonies were intermediate to the two clusters and are designated as unassigned.

Nuclear genetic introgression of Buckfast alleles into ecotypic and non-ecotypic colonies and introgression of alleles from ecotypic and non-ecotypic groups into the Buckfast group was estimated from three iteration each of the $K = 2$ and $K = 3$ models (Table 4). Buckfast nuclear introgression into the ecotype colonies under the $K = 2$ and $K = 3$ models was estimated at 5.83 ± 2.97% and 8.03 ± 2.20%, respectively. Introgression of Buckfast alleles into non-ecotypic colonies was estimated at 8.17 ± 4.12% and 15.83 ± 2.45% under the $K = 2$ and $K = 3$ models, respectively. Introgression of alleles from ecotypic and non-ecotypic *A. m. mellifera* into the Buckfast colonies was estimated at 21.33 ± 1.85% and 8.10 ± 2.21% under the $K = 2$ and $K = 3$ models, respectively.

Discussion

While no single diagnostic morphological or molecular character was found to distinguish the Landes ecotype from the broader *A. m. mellifera* population, the combined morphological and molecular analyses provided a powerful suite of characters for identification of the Landes population. Morphological analysis seemed to be more informative than molecular data for the characterization of the Landes population (both ecotypic and non-ecotypic colonies) from other populations of *A. m. mellifera* yet appears to have little utility in differentiating ecotypic and non-ecotypic Landes colonies. Molecular data were quite useful to distinguish *A. m. mellifera* from imported subspecies in the study area but again provided little information to distinguish among ecotypic and non-ecotypic Landes colonies.

The difficulty in distinguishing ecotypic Landes colonies from non-ecotypic Landes colonies may be derived, in part, from our restrictive definition of ecotype colonies used in this study. Our definition of ecotypic colonies is based on the work of Louveaux et al. (1966) such that only colonies which were found to have the characteristic ecotypic annual brood cycle were designated as ecotype. In fact, it may be that many of the colonies in the non-ecotype Landes group in this study were ecotypic in nature. However, they were not classified as ecotype because interruptions to their brood cycle (due to swarming) during the experiment excluded them from statistical grouping with ecotypic colonies (Strange et al. 2007). Given the results of the present study, it seems likely that our behaviorally defined ecotype and non-ecotype colonies belong to a single population. It may be that the Landes population has a higher frequency of the ecotypic brood trait than could be detected in only one season of brood cycle observation or that within the population the brood cycle trait is not expressed by all colonies. However, this question could only be resolved by gathering multi-year information on brood cycle profiles for the non-ecotypic colonies. For the purposes of a conservation program it is reasonable to use a single year of annual brood cycle data, given the expense and time required to detail brood cycles over multiple years.

The similarity of morphometric results from Landes colonies in the present study and the results obtained by Cornuet et al. (1982) provide further evidence of the

continued presence of the Landes ecotype (Strange et al. 2007). By focusing morphological analysis on the characters identified as informative by discriminant analysis (wing venation and external abdominal characters) the process may be greatly simplified. The statistically significant differences in two morphological characters between the ecotypic and non-ecotypic colonies (width of tomentum light stripe and wing vein angle g18) must be viewed cautiously with regard to their utility for selecting ecotypic colonies. Moritz (1991) demonstrated that selection based upon a few well-differentiated morphological characters did not arrest significant hybridization between two sympatric subspecies when all morphological characters were analyzed. That is, the selection program produced hybrids that went undetected when assays were based on only a few morphological characters typical of the desired parental population. Thus, it is critical to use a broad array of informative characters in the selection of potential breeding stock.

The utility of certain molecular markers is greater than others in distinguishing potential breeder colonies. The mtDNA of the ecotype seems to be limited to the M mitochondrial lineage which is characteristic of *A. m. mellifera*. As the majority of stock imported into the region for permanent culture in the last decade has been the Buckfast hybrid, which is typified by C lineage mtDNA (Perrier et al. 2003), mtDNA analysis can eliminate Buckfast-derived colonies without the need for further molecular or morphometric analysis. That one of the Landes colonies (previously identified by the cooperating beekeeper as "native") was found to have the C1 mitochondrial haplotype (but local morphology and microsatellite allele frequencies) speaks to the utility of mitochondrial DNA screening in conserving the Landes ecotype.

Microsatellite loci were quite variable in their utility to identify Landes *A. m. mellifera* in relation to other populations. While the suite of loci analyzed easily discriminated the Landes population from the Buckfast hybrid and Ille-sur-Tet *A. m. mellifera* populations, we did not detect significant differences in allele frequencies between the ecotypic and non-ecotypic Landes colonies, supporting the conclusion that the two groups represent one population. Although some loci express nearly fixed differences between Buckfast and Landes colonies, there were no such differences between the Landes honey bees and the Ille-sur-Tet honey bees. This indicates that while allele frequency differences exist at the population level, the 10 microsatellite loci we used are not fully diagnostic within *A. m. mellifera*. The microsatellites screened provide an additional method of removing colonies with significant C lineage nuclear DNA, that have non-C lineage mitochondrial haplotypes but better resolution within *A. m. mellifera* would require different loci.

Assignment of individual colonies to population groups based on allele frequencies did not clarify the differences between ecotypic and non-ecotypic colonies. However, this analysis did illustrate introgression between the native Landes honey bees and the introduced Buckfast hybrid at levels similar to those reported by Perrier et al. (2003). Interestingly, increasing the number of populations in the model from $K = 2$ to $K = 3$ marginally increased the fit of the model to the data (based on posterior probabilities) but, instead of separating the ecotypic and non-ecotypic Landes colonies it resulted in a subdivision of the Buckfast colony cluster. Because it seems unlikely that the Buckfast colonies that were maintained in a single apiary were two distinct populations it seems prudent to define the population structure as two groups ($K = 2$), the Landes ecotype and Buckfast bees. The unassigned colonies under this model represent hybrid colonies resulting from Landes bees and Buckfast bees mating. That one ecotypic colony appears to be introgressed by Buckfast alleles may indicate that the brood cycle trait used to describe the Landes ecotype is not lost quickly through introgression or that selection can maintain the trait even in a hybrid population. If it is true that selection can maintain the ecotypic trait in a hybrid population, then it is best to screen all potential breeder stock using morphology and molecular tools in addition to behavioral assays to prevent breeding from introgressed stock.

Using the morphological and molecular data of the present study, it is now possible to design a screening protocol to select colonies for any further annual brood cycle analysis. By targeting selection of potential breeder stock in the Landes, resource managers can reduce the expense of performing difficult ethological analysis on non-native colonies. Until more is known about the prevalence of the brood cycle trait in the Landes, it seems prudent to consider all the locally derived colonies that fall within the Landes cluster in both the molecular and morphological analyses to be a single population targeted for conservation. This will allow the preservation of a broad sample of the genetic diversity within the native Landes honey bees until conservation strategies are refined and more is understood about the nature of the annual brood cycle of the Landes ecotype.

Acknowledgments We thank Ludo Lemaire, Jean-Claude Ducam, Martin Irazoqui and Gilles Fert for providing experimental colonies and graciously donating time, equipment and expertise to the research. We thank the Institut für Bienenkunde in Oberursel, Germany for access to the morphological database. We thank Nicholas Dechamps for assistance in the laboratory with molecular analysis and Marina Meixner for help with data analysis. We thank François Billy and the Parc Régional Natural des Landes de Gascogne for providing staff and funding for this project. Additional funding was provided by the Centre National de la Récherche Scientifique and the Washington State Beekeepers Association.

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