Apidologie 35 (2004) 623–636

DOI: 10.1051/apido:2004061

Original article

Molecular characterisation of indigenous *Apis mellifera carnica* in Slovenia

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(Received 31 October 2002; revised 18 July 2003; accepted 26 March 2004)

Abstract – The genetic structure of *Apis mellifera carnica* bee from Slovenia, collected from 269 localities of ten Slovenian districts, was assessed by mitochondrial and nuclear DNA analyses. The level of genetic variability within and among districts was 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* population. Present study represents the first attempt to characterise indigenous honeybee populations in Slovenia using molecular methods. Furthermore, it indicates that the Carniolan bee from Slovenia still represents an indigenous gene pool within *A. m. carnica*.

***A. m. carnica* / *A. m. macedonica* / mtDNA / COI-COII / microsatellite**

1. INTRODUCTION

Apis mellifera L. is a highly polytypic species. Based on morphometrics, 24 recognised subspecies from the Old World can be grouped in three evolutionary lineages (Ruttner et al., 1978; Ruttner, 1988): M for the west European honey bees, A for the African and C for the north Mediterranean subspecies, which can be further divided into four or five evolutionary lineages (M, A, C, O and Y). The great variety of *A. mellifera* subspecies found around the Mediterranean, fully justifies consideration of this basin as the main gene centre of the species. The group of the central and north-eastern Mediterranean honeybees consists of four closely geographically related subspecies (*A. m. ligustica* Spinola, *A. m. cecropia* Kiesenwetter, *A. m. macedonica* Ruttner and *A. m. carnica* Pollmann; Ruttner, 1988). The Carniolan honey bee, *Apis mellifera carnica* Pollmann, is native to Slovenia and to some regions of the former Yugoslavia, southern Austria, and parts of Hungary, Rumania, and Bulgaria (Ruttner, 1988). *A. m. carnica* expanded from its native range to the central and northern European countries, the United States, Canada and to other parts of the world through the practice of exportation. In contrary to the spread of *A. m. mellifera*, this happened in the second wave of artificial honeybee colonisation, in the second half of the 19th century. The main reasons for the widespread popularity of *A. m. carnica*, included gentle behaviour, good spring build-up of colonies and good summer honey production (Ruttner, 1992).

The analysis of mtDNA has been widely used to study the biogeography of *A. mellifera* subspecies. Five evolutionary lineages of honey bees have been delineated using mtDNA analysis, studying the highly variable COI-COII region (Cornuet et al., 1991; Garnery et al., 1992; Franck et al. 1998, 2001). The variability

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Table I. Details of samples, sample size and COI-COII mtDNA haplotypes, found in each population.

Subspecies	Sample origin	N_1	N_2	COI-COII haplotype
A. mellifera carnica	Slovenia	269	65 (6)	C2C
	Croatia	10	10 (2)	C2C
	Czech republic	9	9 (4)	C1 and C2C
A. mellifera macedonica	Greece	10	10 (3)	C2D
Selected lines				
Hohen Neuendorf	Germany	5	5 (2)	
Buckfast J	Germany	5	5 (3)	C2D
Polen	Poland	5	5 (3)	C2C
K 111	Austria	5	5 (2)	C1
Toulouse	France	5	5 (2)	A8
	Total	323		

N_1 … number of honeybee workers included in microsatellite analysis.
N_2 … number of honeybee workers included in mtDNA analysis; the number of sequenced samples is indicated in parenthesis.

in COI-COII region results from the superimposition of length variation (presence/absence of the P sequence, number of reiterated Q sequences, possible small deletions) and nucleotide substitutions. The shortest haplotypes that lack the P sequence and possess a single Q sequence are characteristic of the C lineage (Garnery et al., 1998). Short size and absence of length variability in the COI-COII region results in a reduced potential for pattern variability in this lineage (Garnery et al., 1993). Only three haplotypes (C1 in *A. m. ligustica*, C2a in *A. m. carnica*, C2b in *A. m. caucasica*) and 5 polymorphic sites (one insertion/deletion and four transitions) have been reported within the C lineage (Franck et al., 2000) and no variation was observed within subspecies. Based on sequence analysis of the ND2 region, however, two haplotypes were found in *A. m. carnica* populations from Austria and Slovenia (Arias and Sheppard, 1996). Accordingly, despite scarce genetic studies it is well documented that *A. m. carnica* differs from other subspecies at mtDNA level. Mitochondrial DNA study of *A. m. carnica* and *A. m. ligustica* samples collected from within areas of endemism also revealed a polymorphism given by the restriction enzyme *Xba*1 (Meixner et al., 1993).

The aim of the present study was to analyse the genetic variability of indigenous *A. m. carnica* in Slovenia, and to follow possible genetic pathways to more or less related honeybee subpopulations in Europe. The area sampled represented only a small fraction of the modern range of *A. m. carnica*. However, historical facts show that the main part of the modern Carniolan bee population originates from the area of the former Austro-Hungarian province of Carniola, nowadays situated in the central part of Slovenia (Mihelic, 1989). One of the goals was also to examine the purity or possible admixture of the Carniolan bee in the region of origin. Nuclear and mitochondrial DNA often display discordant patterns of differentiation in honeybees (Franck et al., 2001), therefore, both of markers (microsatellites and mtDNA) were used to determine the genetic structure of the subspecies *A. m. carnica*.

2. MATERIALS AND METHODS

2.1. Sampling and DNA extraction

A total of 323 honeybee workers were analysed (Tab. I, Fig. 1). One worker represented one colony. 269 honey-bee colonies, one colony per site, separated by at least 2 km, were collected throughout ten Slovenian districts (Koroška, Nova Gorica, Kras, Maribor, Ljubljana, Dolenjska, Bovec, Prekmurje, Gorenjska, Štajerska). In addition, ten samples of the

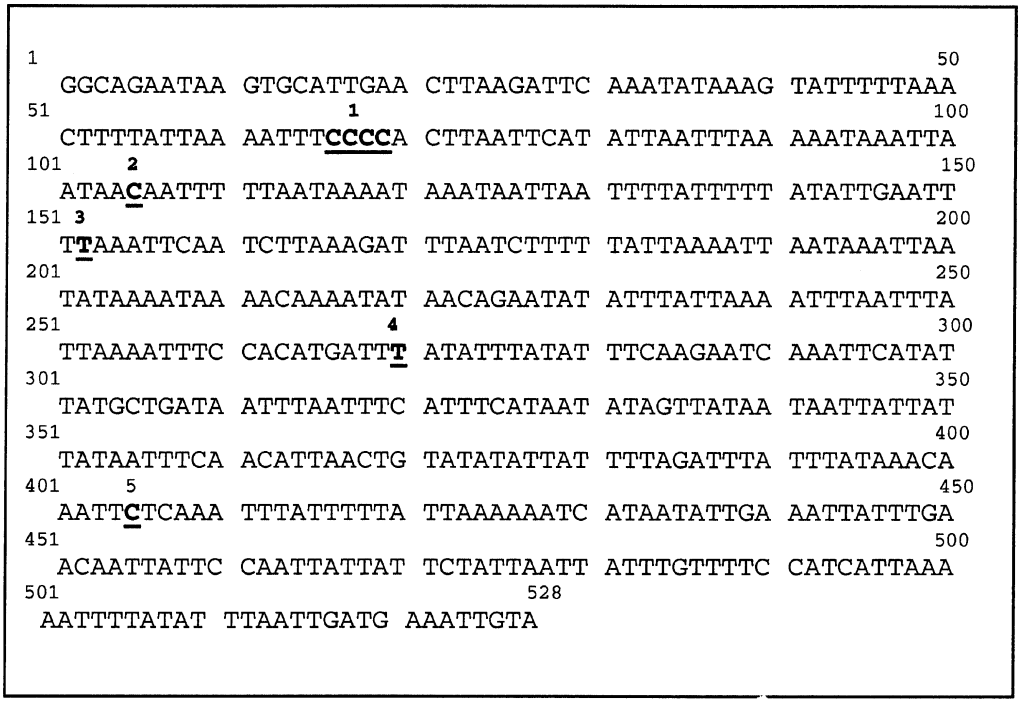

Figure 1. MtDNA COI-COII region sequence of C1 haplotype. Variable nucleotide positions as given in Table II are in bold und underlined.

Carniolan bee from Croatia, nine from the Czech Republic, 10 from Greece (*A. m. macedonica*) and 25 from selected bee lines from the Unije Island breeding program in Croatia, were also included in the analysis. The Unije breeding programme was principally designed to establish the survivorship of bee lines from European institutes without any medication (Büchler et al., 2003). The tested Unije bee lines belong to *A. m. carnica* and others presumably not closely related to the bees from Slovenia. These 25 samples came from named "lines" originating from "Hohen Neuendorf" (Germany) (5), Kirchain "K111" (Germany) (5), "Toulouse" (France) (5), "Polen" (Poland) (5), and from a group of "Buckfast J" bees from Germany (5) and were used to determine its relation towards indigenous Carniolan bee population.

Honeybees were frozen and brought to the laboratory in vials containing 100% ethanol. The abdomen with alimentary tract was removed to avoid cross-amplification, and total DNA was extracted from the worker's head, thoraces and legs according to Beye and Raeder's (1993) protocol. Isolated DNA was used for mitochondrial DNA and microsatellite analysis.

2.2. Mitochondrial DNA analysis

The mtDNA region including the $tRNA^{Leu}$ gene, the COI-COII intergenic region and the 5' end of the COII gene was amplified using a protocol described by Garnery et al. (1993). A fraction of the PCR product was run on 1% agarose gel for size determination. The remaining product was restricted with *Dra*I and restriction fragments were separated on 2.5% agarose. Additionally, 27 samples representing all populations and all RFLP mitotypes were sequenced from both ends using BigDye Terminator Ready Reaction Mix (PE Applied Biosystems). Termination PCR reactions were performed on a programmable thermocycler GeneAmp PCR System 9700 (PE Applied Biosystems), under the following conditions: 10 s denaturation at 96 °C, 5 s annealing at 50 °C and 4 min extension at 60 °C, repeated for 30 cycles. The amplified, fluorescently labelled and terminated DNA was precipitated with sodium acetate and ethanol and analysed on the ABI PRISM 310 automated sequencer. Sequence alignments were done using the computer program Clustal X (Thompson et al., 1997).

2.3. Microsatellite analysis

All honeybee samples were analysed for six microsatellite loci; Ap53 (Franck et al., 1999), A7, A24, A88, A43 (Estoup et al., 1995) and A8 (Franck et al., 1998) following published protocols for amplification.

Aliquots of fluorescently labelled amplified DNA were mixed with formamide and GENESCAN-350 (ROX) Size Standard (PE Applied Biosystems) and genotyped on the ABI Prism 310 Genetic Analyser using the GeneScan^{TM} Analysis Software 2.1.

Population genetic statistics were computed using GENETIX software (Belkhir et al., 1998). Genetic diversity within populations was evaluated by computing allele frequencies and observed and unbiased expected heterozygosity. F-statistics were estimated using the estimators of Weir and Cockerham (1984). Departure from Hardy-Weinberg equilibrium was tested and the F_{is} estimator calculated for each data set. The genetic differentiation between populations was tested by permuting individuals between samples and by computing θ (F_{st} estimator) for each matrix. Factorial Correspondence Analyses (CA) were also conducted using GENETIX software. In each analysis, individuals were first coded according to the presence of the different alleles as: 0 (does not possess allele), 1 (heterozygote), and 2 (homozygote for the allele). Then, composite axes (where each variable contributes differently to the global inertia) that optimised the differences among the analysed individuals were produced. Using CA, different genetic pools can be detected. Furthermore, direct correlation between individuals and alleles can be obtained and discriminant values of each allele can be predicted.

3. RESULTS

3.1. Mitochondrial DNA

The RFLP analysis of the COI-COII mtDNA region was performed. Nevertheless, regarding to the total length of PCR products (~570 and ~640 bp) and to the main distribution of restriction fragments, the COI-COII haplotype could be properly assigned according to already established and verified restriction maps of COI-COII (Franck et al., 2001) to the proper honeybee phylogenetic lineage. To confirm the RFLP results and to define the exact haplotypes, 27 PCR fragments from samples showing different RFLP patterns and belonging to different populations, were sequenced (Tab. I).

Obtained sequences were aligned with published sequences of 42 different haplotypes of honeybees. Except for the samples from the selected line "Toulouse", characterised as haplotype A8, all other determined sequences were of the C phylogenetic lineage, differing in only five already known polymorphic sites (Fig.1, Tab. II). Nevertheless, all haplotypes could not be defined according to already published ones (Franck et al., 2001). In Table II, nucleotides at five variable positions are indicated and new haplotype names are proposed. The haplotypes found in all populations and selected lines are shown in Table I. *A. m. carnica* populations were monomorphic, characterised by a newly found

Table II. Nucleotides at five already described variable positions of COI-COII mtDNA region, differentiating haplotypes of *A. mellifera* C phylogenetic lineage.

		Polymorphic site				
	Haplotype designation	1	2	3	4	5
Previously described haplotypes of the C	C1	CCCC	C	T	T	C
phylogenetic lineage (Franck et al., 2001;	C2A	CCC	A	T	C	T
J.-M. Cornuet, unpublished data)	C2B	CCC	C	A	C	T
	Proposed haplotype designation					
A. m. carnica (Slo, Cro), "Polen", "Hohen Neuendorf"	C2C	CCC	C	T	T	C
A. m. macedonica; "Buckfast J"	C2D	CCC	C	T	C	T
"K 111"	C1	CCCC	C	T	T	C

haplotype, designated as C2C. In the Czech population, haplotype C1 was also found. Samples of *A. m. macedonica* subspecies were also found to be monomorphic for haplotype C2D, differing from *A. m. carnica*-specific haplotype in two C-T transitions (Tab. II).

3.2. Microsatellite loci

All six loci were polymorphic in all samples analysed including the Slovenian group of bees. The Slovenian population of *A. m. carnica* was characterised by possessing 90 alleles at 6 microsatellite loci, where 38 alleles could be described as rare with frequency distributions of less than 5% in all sub-populations (Tab. III). The heterozygosity in the Slovenian bee population was relatively high, ranging from 0.322 for locus A88 to 0.787 for locus Ap53. A deficiency of heterozygotes was detected at two loci in the Slovene populations. Allele frequencies at other loci were in Hardy-Weinberg equilibrium.

The Greek honey bee samples, presumed to belong to the subspecies *A. m. macedonica*, showed less intra-group variability than other groups analysed. They exhibited one-fixed allele (139) at locus A43 and also one allele at a frequency of >90% at two other loci (A24 and A7).

For detection of different genetic pools between all samples, a multidimensional correspondence analysis was performed (Fig. 2). According to the cluster analysis no differentiation among Slovene sub-populations was found; the rare alleles had no influence on individual distribution (data not shown). Furthermore, analysing all populations included in the study, the differentiation was not high, the inertia of the first four axes being 4.05%, 3.85%, 3.32% and 3.12%, respectively.

The close relationship of all *A. m. carnica* samples is evident from Figure 2. The only exception is the group from the Czech Republic, which does not fit completely in this cluster. Even more; some of the individuals were distributed towards samples from some of the selected lines ("Buckfast J" and "Toulouse"). The samples originating from *A. m. macedonica* form a completely separate and very homogenous cluster located on the positive side of the first axis in the cluster analysis diagram. This separation was mainly influenced by three characteristic alleles (215 and 213 at locus Ap53, and 135 at locus A88). Other alleles had no visible influence on sample distribution as they were present in different populations or selected lines in different frequencies.

Pairwise F_{st} values were quite low, but still sufficient to indicate significant differences between some Slovenian districts (Tab. IV). Furthermore, pairwise F_{st} values estimated among all population pairs were relatively high and pointed out highly significant differences between most of the population pairs analysed (Tab. V). As already shown by the cluster analysis, the Croatian and Czech samples did not significantly differ from Slovenian samples (low F_{st} values: 0.01832 and 0.02343, respectively).

Samples from the subspecies *A. m. macedonica* formed the most distinct group among the populations analysed (Fig. 2). Pairwise F_{st} values ranged from 0.15384 to 0.35092, and all values are also highly significant (Tab. V).

Bees that originated from the selected line "Polen" were closely related to populations belonging to *A. m. carnica* subspecies. The "K111" line from Germany and the "Toulouse" selected line did not cluster in the Carniolan group, but they expressed a certain level of similarity to each other.

4. DISCUSSION

According to the results of molecular analysis (mtDNA and microsatellites), the Slovenian honeybee population seems to be very uniform, almost undifferentiated. This result could be underlined by the fact that the same bee population has been the main genetic source for many *A. m. carnica* honeybee groups, which are distributed all over the world or crossbred to form new selected lines with specific characteristics. The bees from Croatia are closely related to the Slovenian bee population, geographically and genetically. Both populations are characterised with only one mtDNA haplotype of COI-COII region, belonging to C phylogenetic lineage and designated as C2C. In the intergenic spacer of the COI and COII mtDNA region, the C evolutionary branch of *A. mellifera* is almost invariant, with only three described haplotypes. In this study, two additional haplotypes were detected, differing from

Table III. Allele frequencies of the six microsatellite loci studied. N: number of colonies studied; H_{exp}: expected heterozygosity according to Hardy-Weinberg equilibrium; H_{obs}: observed heterozygosity.

Locus allele	Population								
	A m carnica			*A m macedonica*	Selected strains				
	Slovenia	Croatia	Czech republic	Greece	Hohen Neuendorf	Buckfast J	Polen	K 111	Toulouse
Ap53									
(N)	258	9	9	10	5	5	5	5	5
245	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
247	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
249	0.008	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
251	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
253	0.006	0.000	0.056	0.000	0.000	0.000	0.000	0.125	0.000
255	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
257	0.006	0.056	0.000	0.000	0.000	0.000	0.000	0.000	0.000
259	0.021	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
261	0.010	0.000	0.000	0.000	0.500	0.100	0.000	0.000	0.000
263	0.002	0.000	0.167	0.000	0.000	0.000	0.000	0.000	0.000
265	0.010	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.100
267	0.012	0.000	0.000	0.000	0.000	0.000	0.000	0.125	0.000
269	0.045	0.000	0.111	0.150	0.100	0.000	0.000	0.000	0.000
271	0.027	0.056	0.056	0.000	0.000	0.000	0.000	0.000	0.000
273	0.048	0.000	0.056	0.000	0.000	0.200	0.000	0.000	0.200
274	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.250	0.000
275	0.035	0.056	0.111	0.000	0.000	0.000	0.000	0.000	0.100
277	0.056	0.056	0.000	0.000	0.000	0.000	0.000	0.000	0.000
279	0.062	0.000	0.167	0.000	0.000	0.000	0.125	0.000	0.200
281	0.078	0.056	0.000	0.000	0.000	0.000	0.500	0.000	0.000
283	0.052	0.056	0.222	0.000	0.000	0.200	0.125	0.000	0.100
285	0.070	0.167	0.056	0.000	0.100	0.000	0.000	0.000	0.000
287	0.080	0.167	0.000	0.000	0.000	0.200	0.000	0.000	0.200
289	0.054	0.000	0.000	0.000	0.000	0.000	0.000	0.375	0.100
291	0.052	0.056	0.000	0.000	0.000	0.000	0.000	0.125	0.000
293	0.048	0.111	0.000	0.000	0.000	0.100	0.000	0.000	0.000
295	0.031	0.000	0.000	0.000	0.100	0.100	0.000	0.000	0.000
297	0.045	0.000	0.000	0.100	0.000	0.000	0.000	0.000	0.000
299	0.039	0.056	0.000	0.250	0.000	0.100	0.000	0.000	0.000
301	0.012	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
303	0.016	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
305	0.016	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
306	0.000	0.000	0.000	0.000	0.000	0.000	0.250	0.000	0.000
307	0.010	0.056	0.000	0.000	0.200	0.000	0.000	0.000	0.000

Table III. Continued.

Locus allele	Population								
	A m carnica			*A m macedonica*	Selected strains				
	Slovenia	Croatia	Czech republic	Greece	Hohen Neuendorf	Buckfast J	Polen	K 111	Toulouse
309	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
311	0.014	0.056	0.000	0.200	0.000	0.000	0.000	0.000	0.000
313	0.008	0.000	0.000	0.250	0.000	0.000	0.000	0.000	0.000
315	0.006	0.000	0.000	0.050	0.000	0.000	0.000	0.000	0.000
323	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
333	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
H exp,	0.952	0.901	0.858	0.800	0.680	0.840	0.656	0.750	0.840
H obs,	0.787	0.889	0.889	0.800	0.800	1.000	0.750	0.500	1.000
A43									
111	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.100
121	0.015	0.000	0.111	0.000	0.000	0.000	0.000	0.000	0.000
123	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.400	0.000
125	0.261	0.050	0.000	0.000	0.600	0.000	0.000	0.000	0.000
126	0.000	0.000	0.278	0.000	0.100	0.200	0.000	0.000	0.700
127	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
139	0.667	0.850	0.611	1.000	0.200	0.800	0.700	0.500	0.000
141	0.047	0.100	0.000	0.000	0.100	0.000	0.300	0.000	0.200
143	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.100	0.000
145	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
H exp,	0.484	0.265	0.537	0.000	0.580	0.320	0.420	0.580	0.460
H obs,	0.489	0.300	0.333	0.000	0.600	0.400	0.200	0.600	0.600
A88									
129	0.000	0.000	0.000	0.000	0.000	0.000	0.100	0.000	0.000
131	0.002	0.000	0.000	0.000	0.000	0.300	0.000	0.100	0.000
135	0.004	0.000	0.000	0.550	0.000	0.000	0.000	0.100	0.000
139	0.070	0.000	0.056	0.050	0.000	0.100	0.100	0.000	0.500
141	0.011	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
143	0.004	0.000	0.000	0.000	0.000	0.200	0.000	0.000	0.000
145	0.081	0.250	0.111	0.100	0.300	0.000	0.000	0.100	0.000
147	0.809	0.700	0.833	0.300	0.700	0.300	0.800	0.700	0.500
149	0.013	0.050	0.000	0.000	0.000	0.100	0.000	0.000	0.000
151	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
H exp,	0.334	0.445	0.290	0.595	0.420	0.760	0.340	0.480	0.500
H obs,	0.322	0.200	0.333	0.800	0.200	1.000	0.400	0.600	1.000
A24									
97	0.000	0.000	0.000	0.000	0.000	0.375	0.000	0.000	0.000
99	0.475	0.450	0.389	0.100	0.800	0.500	0.250	0.500	0.200

Table III. Continued.

Locus allele	Population								
	A m carnica			*A m macedonica*	Selected strains				
	Slovenia	Croatia	Czech republic	Greece	Hohen Neuendorf	Buckfast J	Polen	K 111	Toulouse
101	0.521	0.550	0.611	0.900	0.200	0.125	0.750	0.500	0.800
103	0.004	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
H exp,	0.503	0.495	0.475	0.180	0.320	0.594	0.375	0.500	0.320
H obs,	0.420	0.500	0.333	0.200	0.400	0.750	0.500	0.500	0.400
A8									
151	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
153	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
162	0.000	0.250	0.111	0.000	0.000	0.000	0.000	0.000	0.000
163	0.063	0.000	0.000	0.000	0.000	0.125	0.000	0.000	0.200
165	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
167	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
171	0.011	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.000
172	0.000	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.000
173	0.037	0.000	0.000	0.000	0.000	0.125	0.000	0.000	0.200
175	0.548	0.400	0.556	0.450	0.250	0.250	0.700	0.400	0.300
177	0.052	0.200	0.278	0.350	0.000	0.500	0.000	0.500	0.300
179	0.255	0.050	0.056	0.200	0.500	0.000	0.300	0.100	0.000
181	0.022	0.000	0.000	0.000	0.250	0.000	0.000	0.000	0.000
H exp,	0.626	0.730	0.599	0.635	0.625	0.656	0.420	0.580	0.740
H obs,	0.628	0.800	0.556	1.000	0.750	0.750	0.200	0.600	0.800
A7									
100	0.050	0.000	0.000	0.050	0.000	0.000	0.100	0.000	0.100
101	0.000	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.000
102	0.010	0.000	0.056	0.000	0.000	0.000	0.000	0.000	0.000
104	0.004	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
106	0.200	0.100	0.222	0.000	0.250	0.625	0.000	0.100	0.400
108	0.023	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
110	0.414	0.500	0.500	0.950	0.125	0.250	0.200	0.000	0.200
112	0.210	0.200	0.222	0.000	0.625	0.125	0.400	0.700	0.300
114	0.069	0.150	0.000	0.000	0.000	0.000	0.300	0.200	0.000
116	0.004	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
118	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
120	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
124	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
126	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
128	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
H exp,	0.736	0.675	0.648	0.095	0.531	0.531	0.700	0.460	0.700
H obs,	0.737	0.700	0.889	0.100	0.750	0.750	0.600	0.600	1.000

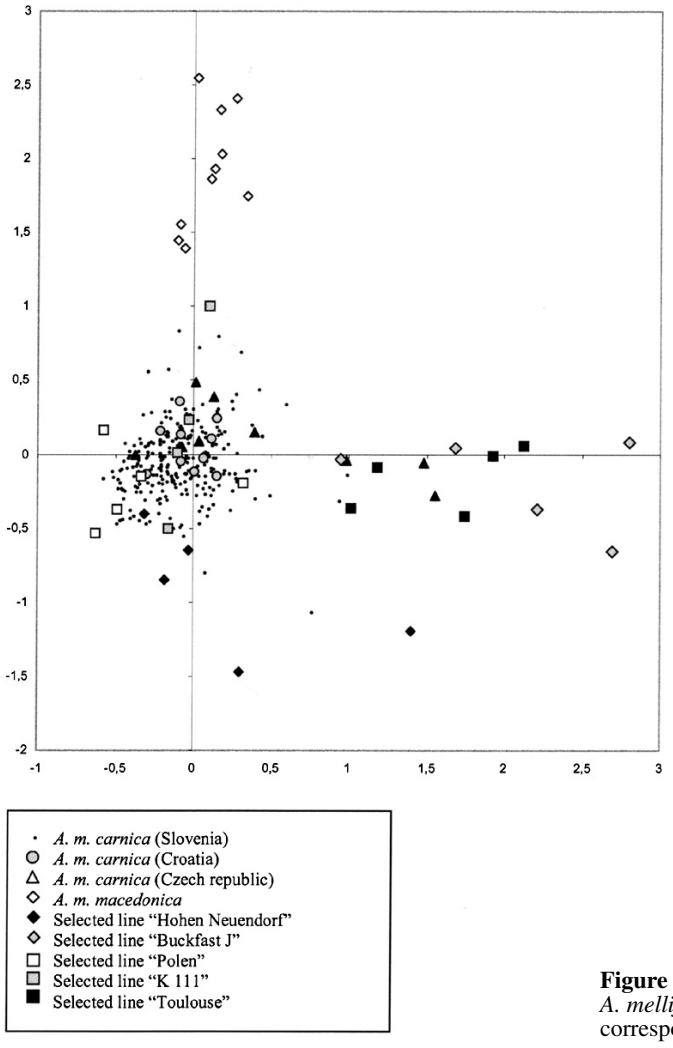

Figure 2. Diagram of distribution of all *A. mellifera* samples analysed according to correspondence analysis.

already described ones only in transitions and one deletion at already known polymorphic sites. The occurrence of haplotypes of different phylogenetic lineages within the *A. m. carnica* populations would indicate secondary admixture (Garnery et al., 1998). Accordingly, populations of *A. m. carnica* in Slovenia and Croatia can be considered to show no indication of the introduction of other subspecies or phylogenetic lineages in this context. Highly variable microsatellites should express the highest level of variability. The only study of microsatellite polymorphism including *A. m. carnica* samples was done by Estoup et al. (1995). Those samples, however, originated from Berlin (Germany), a non-indigenous region within the current distribution of *A. m. carnica*. To complete the knowledge of Carniolan bee genetic structure, our work represents the first study to describing the genetic structure of an *A. m. carnica* population in the centre of its origin. The differentiation among Slovenian subgroups of samples was very low, despite the highly polymorphic markers that were used. The group sampled around Nova Gorica (Western low-land district of Slovenia) was distinct from the groups sampled in Karst, Dolenjska, Prekmurje and Štajerska regions. Bees from

Table IV. Pairwise F_{st} values (above main diagonal) among *A. m. carnica* sub-populations in Slovenia and their significance (in %; below main diagonal) after applying 10000 permutations.

F_{ST}	Koroška	Nova Gorica	Kras	Maribor	Ljubljana	Dolenjska	Bovec	Prekmurje	Gorenjska	Štajerska
Koroška		0.01871	0.00067	0.00373	–0.00615	–0.00414	–0.00340	0.00144	–0.00169	–0.00804
Nova Gorica	99.10		0.00964	0.00257	0.01345	0.01109	0.00719	0.01617	0.00621	0.00075
Kras	ns	95.80		–0.00197	0.00328	0.00213	–0.00326	–0.00378	0.00397	–0.01624
Maribor	ns	ns	ns		–0.00098	0.00448	–0.00768	–0.00334	0.00018	–0.00897
Ljubljana	ns	ns	ns	ns		–0.00591	–0.00537	–0.00298	–0.00330	–0.00788
Dolenjska	ns	98.00	ns	ns	ns		–0.00701	–0.00652	–0.01028	–0.01503
Bovec	ns	ns	ns	ns	ns	ns		–0.01317	–0.00149	–0.01270
Prekmurje	ns	98.00	ns	ns	ns	ns	ns		–0.00477	–0.01535
Gorenjska	ns	ns	ns	ns	ns	ns	ns	ns		–0.01285
Štajerska	ns	ns	0.20	ns	ns	1.50	ns	2.60	ns	

Table V. Pairwise F_{st} values (above main diagonal) among populations or groups of *A. mellifera* analysed and their significance (in %; below main diagonal) after applying 10000 permutations.

F_{st}	1	2	3	4	5	6	7	8	9
1		0.01832	0.02343	0.19020	0.11938	0.12491	0.04006	0.09491	0.17114
2	ns		0.00814	0.15384	0.16820	0.09650	0.05213	0.08456	0.18554
3	ns	ns		0.18584	0.17561	0.10211	0.05688	0.08371	0.10779
4	100	99.70	99.90		0.42741	0.29398	0.26611	0.31794	0.35092
5	99.40	99.40	99.50	99.40		0.20499	0.20156	0.14097	0.23689
6	99.90	98.90	97.80	99.60	96.7		0.20108	0.13055	0.16314
7	ns	ns	ns	99.40	ns	ns		0.09052	0.18647
8	99.50	97.60	97.20	99.40	96.40	97.10	ns		0.16730
9	99.90	98.90	97.20	99.50	97.50	97.40	ns	ns	

1 – *A. m. carnica* (Slovenia)
2 – *A. m. carnica* (Croatia)
3 – *A. m. carnica* (Czech republic)
4 – *A. m. macedonica*
5 – Selected line "Hohen Neuendorf"
6 – Selected line "Buckfast J"
7 – Selected line "Polen"
8 – Selected line "K 111"
9 – Selected line "Toulouse".

Štajerska region were also different from those originating from the Prekmurje (North-eastern region). The basic reason for differences between particular groups of samples can not be explained at present. It is probably a consequence of coincidental admixture of the honeybee population from neighboring regions. *A. m. carnica* occurs close to *A. m. ligustica* in the western low-land district around Nova Gorica and Karst region. A further study including populations from Italy and Austria should be conducted to examine the natural introgression between the distinctive sub-populations. These results will be of particular interest for beekeepers in the future in the case of abolition of the political and market borders between Italy and Slovenia.

Within the Slovenian study area, three morphologically distinct "ecotypes" have been described (Ruttner, 1992); the Alpine, Pannonian and Dalmation. Although we conducted no morphological classification, the three ecotypes should occur in eastern Slovenia. However, bee samples from that area were genetically very uniform and showed similarity to other Slovenian samples.

Based on the loci used in this study, Slovenian bees did not differ from Croatian ones at the nuclear DNA (microsatellite) level. The reason for that could be found in a long border between both countries and the fact that beekeepers from both countries exchange honey bees. The genetic structure of the samples from the Czech Republic indicates a certain level of hybridisation between the native Czech *A. m. mellifera* bee population and *A. m. carnica* derived primarily from Austrian and Slovenian honeybee gene sources (Poklukar, 1999b).

Bees from the Unije selected lines showed a different level of similarity with native Carniolan bee populations. Their genetic composition reflected the controlled admixture of Carniolan bees and bees from other phylogenetic lineages.

The subspecies *A. m. macedonica* belongs to the C phylogenetic lineage of European honey bees (Ruttner, 1988). It was highly differentiated and separated from the *A. m. carnica* samples, based on both mtDNA and microsatellite markers. Following the definition of Hartl and Clark (1997) and in view of the pairwise F_{st} values (0.15384–0.42741), *A. m. macedonica* was highly differentiated from all other populations analysed. Despite their close geographic relationship, *A. m. carnica* and *A. m. macedonica* are also readily differentiated with morphometric analysis (Ruttner, 1988; Arias and Sheppard, 1996).

Modern honeybee populations in many parts of the world are mostly hybrids between native and introduced populations. In some places, they can be considered to reflect rapid migration and successive genetic bottlenecks in localities where they did not exist before (i.e. the New World , Australia, etc.). Genetic studies of endemic honeybee populations are very valuable. In addition to the basic characterisation of specific populations, they can also explain the genetic variability of the newly selected sub-populations and contribute to a wider understanding of the honeybee's genetic structure in general. Honeybee populations in Slovenia express genetic variability selected in nature for thousands of years. This study represents the first attempt to characterise the genetic variability of Slovenian autochtonous bee populations, that remain major sources of indigenous Carniolan bee genes. Bee breeding programmes in Slovenia and honeybee conservation activities currently underway (Poklukar, 1999a) should effectively preserve indigenous *A. m. carnica* as a genetic resource for the future use in bee breeding programs.

ACKNOWLEDGEMENTS

We would like to thank Ministry of Agriculture, Forestry and Food of the Republic of Slovenia who financially supported this project. We would also like to thank all who helped us to collect bee sample. We would like to express our special thanks to Prof. G. Theophilidis, from the Aristotle University of Thessaloniki, Greece, Ing. D. Titera from Bee institute Dol, Prague, Czech Republic and Prof. Dr. N. Kezic from the Zagreb University (Croatia). We are very thankful to Prof. J.-M. Cornuet, who kindly provided the aligned sequences of 42 published haplotypes of *Apis mellifera* COI-COII mtDNA region. We are grateful to Prof. Peter Dovè for helpful comments on an earlier version of the manuscript.

Résumé – Caractérisation moléculaire de l'abeille indigène *Apis mellifera carnica* en Slovénie. La morphométrie a permis de reconnaître dans l'Ancien Monde vingt quatre sous-espèces qui se regroupent en trois lignées évolutives : la lignée M

pour les sous-espèces d'Europe de l'ouest, la lignée A pour les africaines et la lignée C pour les nord-méditerranéennes. L'abeille carnolienne, *Apis mellifera carnica* Pollmann, est l'une des sous-espèces de la lignée C. Elle est originaire de la Slovénie, de certaines régions de l'ancienne Yougoslavie, du sud de l'Autriche et de certaines régions de Hongrie, Roumanie et Bulgarie.

Le but de l'étude était d'analyser la variabilité génétique des populations indigènes d'*A. m. carnica* en Slovénie. Les profils de différentiation génétique sont souvent différents lorsqu'on compare la variabilité de l'ADN nucléaire à celle de ADN mitochondrial chez l'abeille ; nous avons donc utilisé deux types de marqueurs (microsatellites et ADNmt) pour déterminer la structure génétique d'*A. m. carnica*.

Au total 323 ouvrières ont été analysées (Tab. I) provenant de 269 colonies d'abeilles, à raison d'une par site, séparées l'une de l'autre d'au moins 2 km et réparties dans les 10 régions de Slovénie. Des échantillons supplémentaires d'*A. m. carnica* provenant de Croatie et de la République Tchèque ont été inclus dans l'analyse. Des échantillons de Grèce (*A. m. macedonica*) et des lignées d'abeilles sélectionnées du programme de sélection de l'île d'Unije en Croatie ont été utilisés comme groupes externes.

Une analyse par RFLP de la région COI-COII de l'ADNmt a été faite. En outre, les échantillons qui présentaient des profils RFLP différents ont été séquencés. A l'exception des échantillons de la lignée sélectionnée « Toulouse » caractérisés par l'haplotype A8, toutes les autres séquences déterminées étaient de la lignée phylogénétique C et ne différaient que par 5 sites polymorphes déjà connus. Néanmoins nous n'avons pas pu définir tous les haplotypes présents en fonction des haplotypes déjà publiés. Nous caractérisons un nouvel haplotype, désigné par C2C, au sein d'*A. m. carnica*. En outre un haplotype précédemment décrit (C1) a été trouvé dans la population tchèque. Les échantillons d'*A. m. macedonica* étaient monomorphes pour l'haplotype C2D (Tab. II).

Tous les échantillons ont été analysés à 6 locus microsatellites, Ap53, A7, A24, A88, A43 et A8, selon les protocoles décrits pour l'amplification de ces locus. Au total 90 allèles ont été observés sur l'ensemble des 6 locus dans l'échantillon slovène d'*A. m. carnica*. L'hétérozygocie dans les populations slovènes est relativement élevée, comprise entre 0,322 et 0,787 (Tab. III). Les valeurs de F_{st} entre chaque groupe d'échantillons slovènes sont inférieures à 0,0871, ce qui indique que la population d'abeilles carnoliennes est homogène dans cette région (Tab. IV, Fig. 1). Les échantillons slovènes ne différaient pas non plus des échantillons croates. La sous-espèce *A. m. macedonica* était par contre bien différenciée et séparée d'*A. m. carnica* par l'analyse de l'ADNmt et des microsatellites.

Cette étude représente la première tentative pour caractériser la variabilité génétique des populations d'abeilles slovènes autochtones. La même population d'abeilles a constitué la source génétique principale pour de nombreux groupes d'*A. m. carnica* qui ont été répartis dans le monde entier ou croisés afin de créer de nouvelles lignées sélectionnées avec des caractéristiques spécifiques. Les programmes de sélection de l'abeille en Slovénie et les activités de conservation de l'abeille actuellement en cours (Poklukar, 1999a) devraient préserver de façon efficace l'abeille *A. m. carnica* indigène comme ressource génétique pour une utilisation future dans des programmes de sélection.

***Apis mellifera carnica* / *Apis mellifera macedonica* / génétique populations / ADNmt / microsatellite / COI-COII**

Zusammenfassung – Molekulare Charakterisierung der in Slowenien einheimischen *Apis mellifera carnica*. *Apis mellifera* ist eine ausgeprägt polytypische Art. Basierend auf morphometrischen Analysen können die 24 anerkannten Subspezies der Alten Welt in drei evolutive Linien gruppiert werden: die Linie M mit den westeuropäischen, Linie A mit den afrikanischen und Linie C mit den nordmediterranen Unterarten. Die Kärnter Honigbiene, *Apis mellifera carnica* Pollmann, ist eine der Unterarten der phylogenetischen Linie C. Sie kommt nativ in Slowenien, einigen Regionen des früheren Jugoslawien, Südösterreich und in Teilen von Ungarn, Rumänien und Bulgarien vor.

Ziel der vorliegenden Studie war es, die genetische Variabilität der in Slowenien einheimischen *A. m. carnica* zu untersuchen. Da die nukleare und mitochondriale DNA bei Honigbienen oft abweichende Muster der Differenzierung zeigen, wurden beide Markertypen (Mikrosatelliten und mtDNA) eingesetzt, um die genetische Struktur von *A. m. carnica* aufzuklären.

Insgesamt wurden 323 Arbeiterinnen untersucht (Tab. I). Diese waren aus 269 Bienenvölkern aus jeweils einem Volk pro Standort gesammelt worden. Die Standorte lagen dabei mindestens 2 km auseinander und waren über zehn Distrikte Sloweniens verteilt. Zusätzliche Proben stammten aus Kroatien und der Tschechischen Republik. Bienenproben aus Griechenland (*A. m. macedonica*) und selektionierte Linien des Zuchtporgramms auf der Insel Unije in Kroatien lieferten die Aussengruppen. Die COI-COII mtDNA Region wurde mittels RFLP-Analyse untersucht, wobei Proben mit abweichenden RFLP-Mustern sequenziert wurden. Ausser den Proben der Zuchtlinie „Toulouse", für die der Haplotyp A8 charakteristisch ist, fielen alle anderen Sequenzen in die phylogenetische Linie C und unterschieden sich lediglich in fünf bereits als polymorph bekannten Orten. Nichtsdestotrotz konnten nicht alle Haplotypen den bereits bekannten zugeordnet werden. Wir beschreiben einen als C2C bezeichneten neuen Haplotyp innerhalb von *A. m. carnica*. Zusätzlich fanden wir einen bereits beschriebenen Haplotyp (C1) in der tschechischen Population. Proben von *A. m. macedonica* erwiesen sich als monomorph für Haplotyp C2D (Tab. II).

Alle Bienenproben wurden hinsichtlich der Mikrosatellitenloci Ap53, A7, A24, A88, A43 und A8 mittels der jeweils beschriebenen Amplifikationsprotokolle untersucht. Die slowenischen Proben wiesen an den 6 Mikrosatellitenloci insgesamt 90 Allele auf. Der Heterozygotiegrad in der slowenischen Bienenpopulation erwies sich mit 0,322 bis 0,787 als relativ hoch (Tab. III). Der Differenzierungsgrad zwischen Untergruppen der slowenischen Proben war sehr niedrig, mit paarweisen F_{st}-Werten unter 0,01871. Dies weist auf eine sehr homogene Populationsstruktur der Kärtnerbiene in dieser Region hin (Tab. IV, Abb. 1). Entsprechend den Ergebnissen unserer molekularen Analysen ist die slowenische Honigbienenpopulation sehr homogen und nahezu undifferenziert. Wir fanden keine Unterschiede zwischen den slowenischen und den kroatischen Proben. Im Gegensatz hierzu erwiesen sich die Proben der Subspezies *A. m. macedonica* sowohl in der mtDNA als auch in der Mikrosatellitenanalyse als deutlich differenziert und getrennt von den *A. m. carnica* Proben. Diese Untersuchung stellt den ersten Ansatz dar, die genetische Variabilität der autochtonen Bienenpopulation in Slowenien zu erfassen, die eine Hauptquelle der einheimischen Kärtnerbiene darstellt. Gegenwärtig in Slowenien laufende Bienenzuchtprogramme und Aktivitäten zum Schutz der Honigbienen (Poklukar, 1999a) sollten effektiv dazu beitragen, die einheimische *A. m. carnica* als genetische Ressource für künftige Bienenzuchtprogramme zu erhalten.

Dieses Ergebnis wird dadurch unterstrichen, dass genau diese Bienenpopulation die hauptsächliche genetische Quelle der vielen *A. m. carnica* Gruppen darstellt, die weltweit Verbreitung gefunden haben und verschiedentlich eingekreuzt wurden, um neue Zuchtlinien mit bestimmten Merkmalen hervorzubringen. Die slowenische Bienenpopulation ist immer noch die Hauptquelle der Gene der einheimischen Kärtnerbiene. Die in Slowenien laufenden Konservierungsprogramme sollten damit effektiv die einheimischen *A. m. carnica* Landrassen und die genetischen Ressourcen für künftige Bienenzuchtprogramme erhalten.

***Apis m. carnica* / *Apis m. macedonica* / mtDNA / COI-COII / Mikrosatelliten**

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