MOLECULAR CHARACTERISATION OF INDIGENOUS *APIS MELLIFERA CARNICA* POLLMANN IN SLOVENIA

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Abstract

*Carniolan bee (*Apis mellifera carnica*) and C phylogenetic lineage of honeybees as a whole, is genetically poorly studied, therefore, the genetic structure of Carniolan bee population from Slovenia has been assessed by mitochondrial and nuclear DNA analyses. Honeybees were collected from 269 localities in Slovenia; bee samples from Greece, Czech Republic, Croatia, Germany and France were included in the analysis as out-groups. Slovenian samples are characterized with a low level of genetic differentiation in COI and COII region of mtDNA. They were fixed for one newly found mtDNA haplotype, designated as C2C. The same haplotype was found in Croatian and Polish out-group and in some German and some Czech bee samples. Low level of variability of the Carniolan bee population in Slovenia was observed also for all six microsatellite loci studied, showing very homogenous structure of indigenous Slovenian bee population. Populations from Croatia and Czech Republic did not differ significantly from Slovenian one based on microsatellite analysis. On the other hand, high genetic differentiation was observed towards the* A. m. macedonica *population, also expressing specific mtDNA haplotype, designated as C2D. The already described C1 haplotype was found in Austrian and in some samples of Czech out-group. The only haplotype of non-C phylogenetic lineage origin was characteristic for French sample of bees (A8 haplotype). The results as such indicate that Carniolan bee from Slovenia still represents one of the major sources of the* A. m. carnica *indigenous gene pool.*

Keywords: *biodiversity / Carniolan bee / genetic analyses*

Introduction

Apis mellifera 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): European Honey bees (M), African (A) and north Mediterranean (C lineage). The group of the central and north-eastern Mediterranean honeybees consists of five closely geographically related subspecies (*A.m. sicula* Montagano, *A. m. ligustica* Spinola, *A. m. cecropia* Kiessewetter, *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* has been spread from its native districts to the central and northern European countries, USA and Canada. The main causes for such a process were gentle behaviour of *A. m. carnica* towards beekeepers, good spring honey production and good production of summer honey originated from honeydew of coniferous trees.

The analysis of mtDNA has become widely used approach in studying the biogeography of the *A. mellifera* subspecies. Three evolutionary lineages of honey bees have also been found studying highly variable COI-COII region (CORNUET et al., 1991). The variability 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. Only three haplotypes (C1 in *A. m. ligustica*, C2a in *A. m. carnica*, C2b in *A. m. caucasica*) were reported within the C phylogenetic lineage (FRANCK et al., 2000) and no variation was observed within the subspecies. Furthermore, also highly variable microsatellite loci are being increasingly used in population genetics studies of *A. mellifera* subspecies (FRANCK et al., 2000; DE LA RÚA et al., 2001). The final general structure of the species with its three main evolutionary branches has been revealed also analysing microsatellites (ESTOUP et al., 1995; FRANCK et al., 1998).

The aim of the present study is therefore, to characterise and to analyse the genetic variability of indigenous *A. m. carnica* in Slovenia, and to follow possible genetic pathways to more or less related honeybee sub-populations in Europe.

Material and Methods

A total of 323 honeybee workers were analysed. 269 honey-bee colonies were collected throughout Slovenia. Ten samples of the Carniolan bee from Croatia were included in the analysis. In addition, nine bee samples 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 used as outgroups (Table I). 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. The mtDNA region, including the tRNALeu 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). Restriction with D*ra*I on 119 samples and sequencing of 27 samples were performed. 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). Population genetics statistics were computed using the GENETIX software (BELKHIR et al. 1998).

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)	C2C
Buckfast J	Germany	5	5 (3)	C2D
Poland	Poland	5	5 (3)	C2C
K 111	Austria	5	5 (2)	C1
Toulouse	France	5	5 (2)	A8
	Total	323		

N1 … number of honeybee workers included in microsatellite analysis
N2 … number of honeybee workers included in mtDNA analysis; the number of sequenced samples is indicated in parenthesis

Results and Discussion

Mitochondrial DNA

With an exception of samples from selected line from France, all other samples were characterized with mtDNA sequences of the C phylogenetic lineage (Table I). All haplotypes found in this study could not be defined according to already published ones. Two new haplotypes of the C phylogenetic lineage were detected, differing from already described haplotypes only in different transitions on already known polymorphic sites (Table II). *A. m. carnica* populations were monomorphic, characterised with newly found haplotype, designated as C2C. Samples of the *A. m. macedonica* subspecies were also found to be monomorphic for new haplotype C2D.

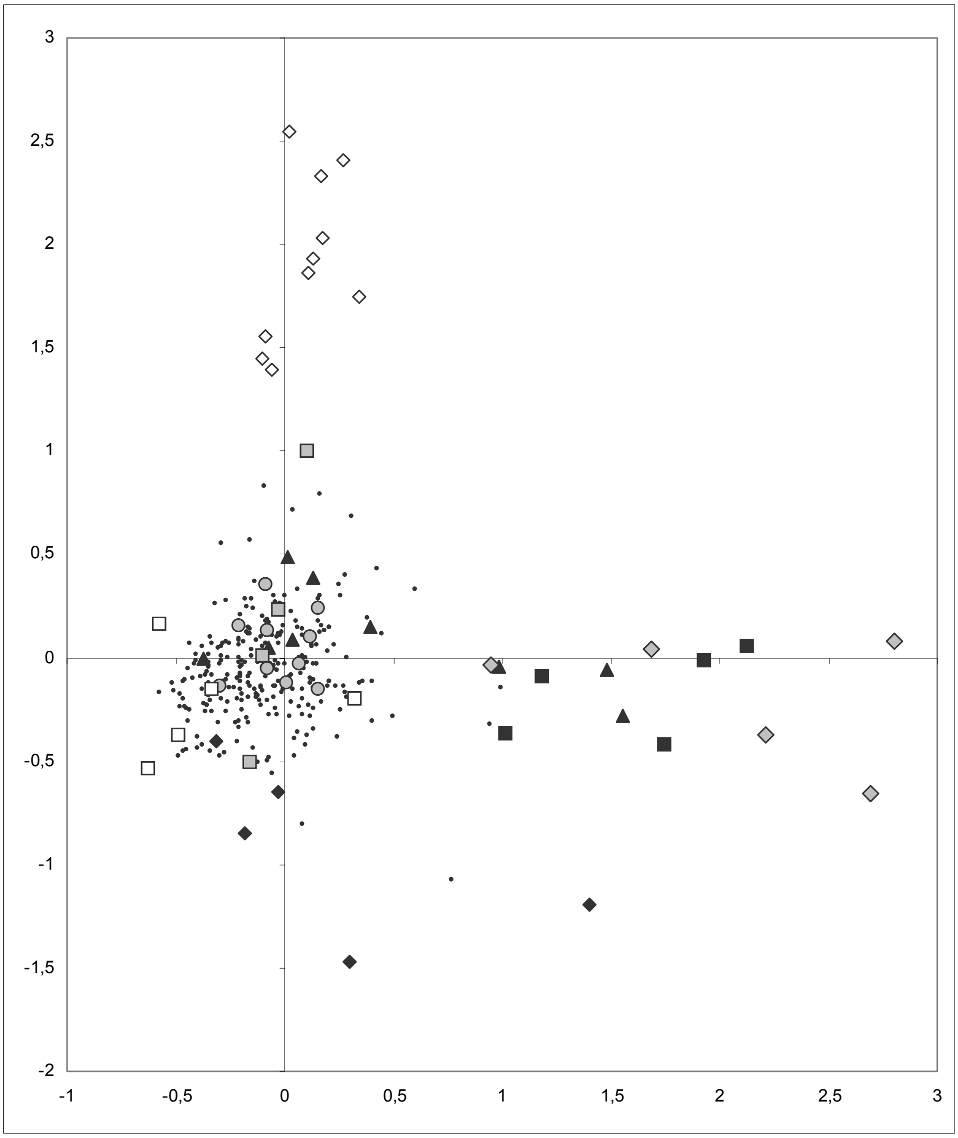
Table II

Nucleotides at five already described variable positions of COI-COI 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 phylogenetic lineage (Franck *et al.*, 2001; J.-M. Cornuet, pers. com.)	C1	CCCC	C	T	T	C
	C2A	CCC	A	T	C	T
	C2B	CCC	C	A	C	T
	Proposed haplotype designation					
A. m. carnica (Slo, Cro), “Poland”, „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

Microsatellites

All six microsatellite loci were polymorphic in all samples analyzed. For detection of different genetic pools between all samples analysed, the multidimensional correspondence analysis was performed. According to the cluster analysis no differentiation among Slovene sub-populations was found. Clustering of all *A. m. carnica* samples from Slovenia and Croatia is evident (Figure 1). The samples of *A. m. macedonica* form completely separate and very homogenous cluster. Bees from the selected lines showed different relationship towards native Carniolan bee populations. Their genetic composition reflects the controlled admixture of Carniolan bees and bees of other origin in the past.



A. m. carnica (Slovenia)
A. m. carnica (Croatia)
A. m. carnica (Czech republic)
A. m. macedonica
Selected line “Hohen Neuendorf”
Selected line “Buckfast J”
Selected line “Poland”
Selected line “K 111”
Selected line “Toulouse”

Figure 1 - Diagram of distribution of all A. mellifera *samples analysed according to correspondence analysis*

Conclusion

According to the results of molecular analysis, the Slovenian and Croatian honeybee population seems to be very uniform, almost undifferentiated. Accordingly, populations of *A. m. carnica* in Slovenia and Croatia could be considered as a very native with no introduction of other subspecies or phylogenetic lineages.

REFERENCES

Belkhir K., Borsa P. (1998), GENETIX, logiciel sous WindowsTM pour la génétique des populations. http://www.univ-montp2.fr/~genetix/genetix.htm, Laboratoire Génome et Populations, CNRS UPR 9060, Université Montpellier II, Montpellier (France)

Beye M., Raeder U. (1993), Rapid DNA preparation from bees and %GC fractionation, *BioTechniques* 14, 372-374

Cornuet J.-M., Garnery L., Solignac M. (1991), Putative origin and function of the intergenic region COI and COII of *Apis mellifera*: mitochondrial DNA, *Genetics*, 1128, 393-403

De la Rúa P., Galian J., Serrano J., Moritz R.F.A. (2001a), Genetic structure and distinctness of *Apis mellifera* L. populations from the Canary Islands, *Mol. Ecol*. 10, 1733-1742

Estoup A., Garnery L., Solignac M., Cornuet J.-M. (1995), Microsatellite variation in honey bee (*Apis mellifera* L.) populations: hierarchical genetic structure and test of the infinite allele and stepwise mutation models, *Genetics* 140, 679-695

Franck P., Coussy H., Le Conte Y., Solignac M., Garnery L., Cornuet J.-M. (1999), Microsatellite analysis of sperm admixture in honeybee, *Insect Mol. Biol*. 8, 419-421

Franck P., Garnery L., Celebrano G., Solignac M., Cornuet J.-M. (2000), Hybrid origins of honeybees from Italy (*Apis mellifera ligustica*) and Sicily (*A. m. sicula*), *Mol. Ecol*. 9, 907-921

Franck P., Garnery L., Loiseau A., Oldroyd B. P., Hepburn H. R., Solignac M., Cornuet J.-M. (2001), Genetic diversity of the honeybee in Africa: microsatellite and mitochondrial data, *Heredity* 86, 420-430

Franck P., Garnery L., Solignac M., Cornuet J.-M. (1998), The origin of west European subspecies of honeybees (*Apis mellifera*): New insights from microsatellite and mitochondrial data, *Evolution* 52, 1119-1134

Garnery L., Solignac M., Celebrano G, Cornuet J.-M. (1993), A simple test using restricted PCR-amplified mitochondrial DNA to study the genetic structure of *Apis mellifera* L., *Experientia* 49, 1016-1021

Ruttner F. (1988), Biogeography and taxonomy of honeybees, Springer-Verlag, Berlin, Heidelberg, 284 pp

Ruttner F., Tassencourt L., Louveaux J. (1978), Biometrical-statistical analysis of the geographic variability of *Apis mellifera* L., *Apidologie* 9, 363-381