Journal of Microbiology, Biotechnology and Food Sciences

Paál et al. 2013 : 2 (Special issue 1) 1517-1525

REGULAR ARTICLE

MICROSATELLITE ANALYSIS OF THE SLOVAK CARNIOLAN HONEY BEE (*APIS MELLIFERA CARNICA*)

*Dušan Paál [1], Ján Kopernický [2], Jaroslav Gasper [2], Dušan Vašíček [2], Katarína Vašíčková [2], Mária Bauerová [1], Miroslav Bauer * [1,2]*

Address: Miroslav Bauer, [1]Constantine the Philosopher University in Nitra, Department of Botany and Genetics, Nábrežie mládeže 91, 949 74 Nitra, Slovak Republic.

[2]Animal Production Research Centre Nitra, Hlohovecká 2, 951 41 Lužianky, Slovak Republic.

*Corresponding author: bauer@cvzv.sk

ABSTRACT

The aim of this study was the selection and testing of suitable microsatellite markers for evaluation of the Slovak carniolan honey bee, particularly the population structure, genetic diversity, breed assignment and paternity testing of honey bee queens in Slovakia. Fourteen microsatellite markers running in two multiplex PCR reactions have been tested on 40 randomly selected workers and queens and further verified by PIC index, expected heterozygosity (H_E) and observed heterozygosity (H_O). Chi-squared test of goodness of fit ($\alpha = 0,05$) was used to check the Hardy-Weinberg equilibrium (HWE) of genotype for each marker. For the comparison tests the workers of *A. mellifera mellifera x ligurica, A. mellifera macedonica and A. mellifera iberica* were analyzed, using the same set of markers. We identified a total of 123 alleles in the Slovak carniolan honey bee samples, with the mean value of 8,78 allele per locus. Eleven markers showed the PIC value greater than 0,5 and thus were highly informative. The mean value of expected heterozygosity H_E for all loci was 0,705 ± 0,15, the mean value of observed heterozygosity H_O was 0,704 ± 0,18. The frequencies of genotypes for most tested markers were in HWE.

Keywords: carniolan honey bee, microsatellite markers, multiplex PCR

INTRODUCTION

The most common honey bee in Slovakia is the autochtonous carniolan breed (*Apis mellifera carnica*), originating from mountainous northwestern region of Slovenia (Kransko). This breed is well adapted to climate conditions in Slovakia, showing a good viability, resistance to diseases and acceptable honey production. From the alpine type, originating from the area of the current Austria, the best known and the most extended tribes are Sklenar, Troiseck and Peschetz, from carpathian type originates Vučko and from autochtonous tribes originate Tatranka, Devínčanka, Šarišanka, Vigor, Košičanka and Vojničanka (**Chlebo, 2003**).

Population of the Slovak carniolan honey bee can be negatively influenced by individual import of allochtonous breeds of bees. These allochtonous breeds could be the source of a new pathological strains of microbes and parasites. Since the traditional reservoirs of the carniolan honey bee in Austria and in northern Slovenia have been decimated due to mass extinction of hives in the eighties of the last century and supplemented by honey bees of dissimilar origin, it is necessary to maintain the gene pool of the native Slovak carniolan honey bee population (**VÚŽV, 2012**).

The long-term method used for species and interspecies variability studies is morphometry, which is based on comparison of quantitative parameters, whose value can be expressed by spatial measures. For the preservation of the gene pool of native carniolan honey bee it is necessary to extend traditional methods of phenotype evaluation on the basis of morphological signs, with modern molecular methods, based on screening of genetic markers, which are specific for each breed. **Sheppard and McPheron (1986)** investigated the genetic variability of honey bees from western Czechoslovakia using polymorphic enzyme electrophoresis. In the past years, many DNA markers, which make it possible to observe the influence of introduced genes on native honey bee population, have been developed (**Estoup et al., 1995; Vačkář et al., 1995**). For these purposes, the most suitable markers are considered microsatellites which have co-dominant heredity, great number of alleles per locus and slow mutation speed (**Estoup et al. 1993**).

Microsatellites are short tandem repeats (STR) of 1 – 6 base pairs distributed randomly through the whole genome of Eucaryota. The number of repeats is individual-specific and the whole microsatellite locus consists of several hundred base pairs (**Brooker, 2012**). Microsatellites represent suitable genetic markers for the origin verification and the identification of individuals, as well as for the testing of genetic variability of breeds and

populations. Nowadays, the analysis of microsatellites is recommended by ISAG/FAO Commission as a standard procedure for animal genetic resources conservation and animal paternity testing (**FAO, 2011**).

MATERIALS AND METHODS

Sampling

We analyzed a total of 83 samples of honey bee. Forty samples of *A. mellifera* carnica were provided by the Institute of Apiculture, Liptovský Hrádok and by the beekeepers and breeders Vladimír Sokol, Raslavice, Ján Bojtim, Dargov, Arpád Marušinec, Bátorové Kosihy. For the comparison tests, 11 samples of *A. mellifera mellifera x ligurica* originated from Liptovský Hrádok (Slovakia), 10 samples of *A. mellifera macedonica* originated from Chalkidiki region (Greece). 10 samples of *A. mellifera iberica* originated from Cieza region (Spain) and 12 samples of *A. mellifera iberica* coming from La Union region (Spain) were sampled.

DNA isolation

DNA was isolated from anterior leg after rinsing in 96% ethanol and sterile bi-distilled water using NUCLEOSPIN® TISSUE isolation kit (Macherey-Nagel, Düren, Germany) according to the manufacturer´s procedure. The concentration and purity of isolated DNA were determined spectrophotometrically by GENEQUANT II (Pharmacia Biotech, Piscataway, USA) at 260 nm and 280 nm wavelength.

Microsatellite markers

We tested 14 microsatellite markers, 4 of which are linked together on chromosome 13 (HB-THE-01, HB-THE-02, HB-THE-03, HB-THE-04) and 3 markers on chromosome 16 (HB-C16-01, HB-C16-02, AC006). The remaining 7 markers (UN351, A079, A113, A024, A107, A007, AP043) are unlinked (**Shaibi et al., 2008**).

DNA amplification

Amplification of DNA was performed in 16 µl reactions using the QIAGEN MULTIPLEX PCR KIT (Qiagen, Hilden, Germany). PCR reaction was done on DNA ENGINE PTC 200 THERMOCYCLER (MJ Research, Waltham, USA). Multiplex I PCR program: 95 °C for 15 min; 30 cycles (94 °C for 30 s; 59 °C for 90 s; 72 °C for 60 s); final incubation step at 60 °C for 30 min. Multiplex II PCR program: 95 °C for 15 min; 30 cycles (94 °C for 30 s; 60 °C for 90 s; 72 °C for 60 s); final incubation step at 60 °C for 30 min.

Fragment analysis

The PCR products were diluted 15 – 20 times with sterile MILLIQ (Merck Millipore, Billerica, USA) water. Then 1,2 µl of diluted PCR product was mixed with 0,3 µl of GENESCAN – 500 LIZ SIZE STANDARD (Applied Biosystems, Paisley, UK), 10,5 µl of deionized formamide (Applied Biosystems, Paisley, UK) and denatured for 3 min. at 95°C. After 5 min. of cooling on ice the mixture was analyzed on ABI PRISM 310 (Applied Biosystems, Carlsbad, USA) using the GeneScan module. The fragment analysis was performed using the GeneMapper software, version 4.0.

Statistics

Obtained data were statistically analyzed with the PowerMarker 3.25 (**Liu – Muse, 2005**). Hardy - Weinberg equilibrium, expected heterozygosity and observed heterozygosity were tested by Chi-square test ($\alpha = 0, 05$).

RESULTS AND DISCUSSION

Opposite to other species of farm animals (cattle, horses, sheeps, goats) there are not specified and recommended sets of microsatellite markers, so-called FAO/ISAG panels for testing of paternity and genetic diversity of honey bee (**FAO, 2011**). Therefore it is necessary to choose suitable microsatellite markers from several hundred published markers (**Solignac et al., 2007**) and optimize appropriate multiplex PCR reactions.

By testing of 14 microsatellite markers in two multiplex PCR reactions in the set of 40 samples of the Slovak carniolan honey bee, we detected very different number of alleles for tested markers. The highest number of alleles was recorded for the marker HB-C16-02 and

the lowest number of 2 alleles showed the marker HB-THE-04. The summarized results are shown in Fig. 1. Overall we identified 123 alleles by using 14 microsatellite markers with the average number of 8,78 allele per locus. Generally, it is recommended to use markers with at least 5 alleles for genetic variability screening, paternity testing and origin determination. In our experiments, there are 3 markers that don't meet this recommendation (AC006, A024 a HB-THE-04). We observed similar number of alleles for used markers also in the case of the Buckfast, the Macedonian and the Iberian honey bees testing.

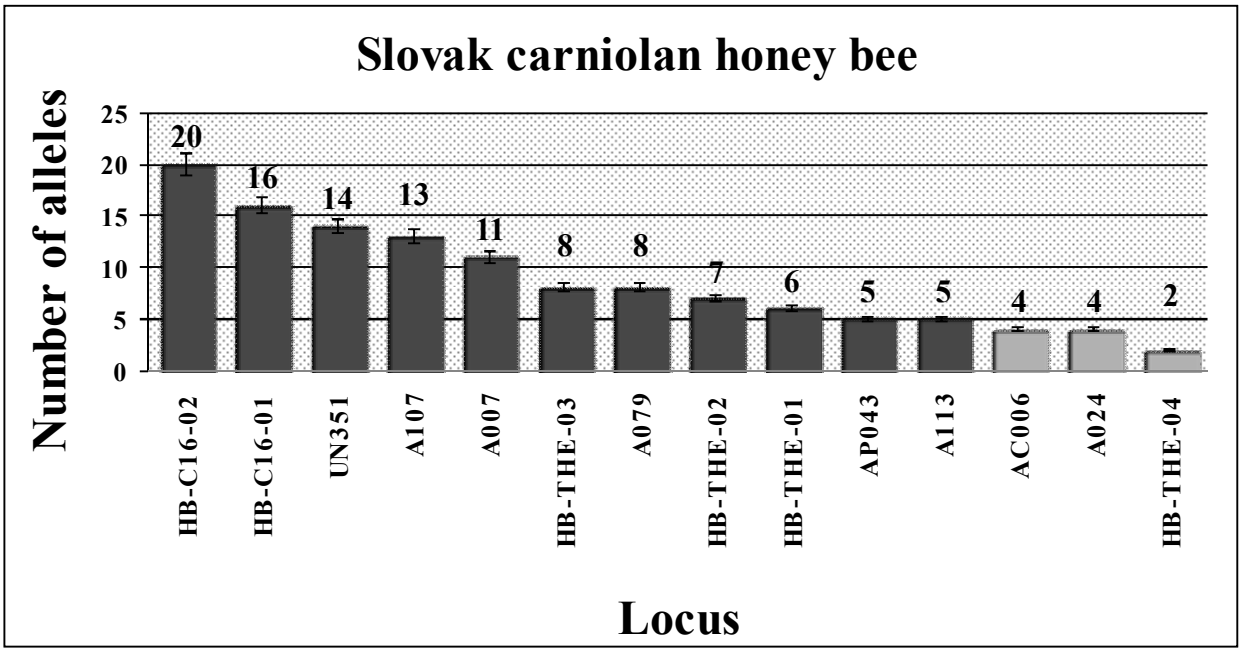

Figure 1 Number of identified alleles for each microsatellite marker. Error bars with standard error are also shown

Legend: number of alleles ≥ 5
number of alleles < 5

Analyzing a greater set of individuals in the population of the Slovak carniolan honey bee, it is possible to expect an increase of number of alleles for markers AC006 and A024. Marker HB-THE-04 has shown a low number of alleles also in other studies (**Shaibi et al., 2008**), therefore this marker is not considered suitable neither for practical use by screening of genetic variability, nor for origin verifying.

The information value of tested markers renders the PIC index (Polymorphism Information Content). This PIC value is a measure of polymorphism of the marker within an interval ⟨0 ; 1). Highly informative marker has the PIC ≥ 0, 5. Reasonably informative marker has the value 0, 5 > PIC ≥ 0, 25. In the case of briefly informative marker, the PIC value is PIC < 0,

25 (**Botstein, 1980**). Fig. 2 shows the PIC values for tested markers in a set of 40 individuals of the Slovak carniolan honey bee. The highest value (0,920) has the marker HB-C16-02 and the lowest value (0,344) belongs to the marker HB-THE-04, which has only 2 alleles. From the data in Fig. 2 we can conclude that 11 of 14 tested markers are highly informative and represent good source set for population studies as well as for verification of the origin of the Slovak carniolan honey bee. Only 3 markers (AC006, A113 a HB-THE-04) have the PIC value lower than 0, 5 and represent a reasonably informative markers.

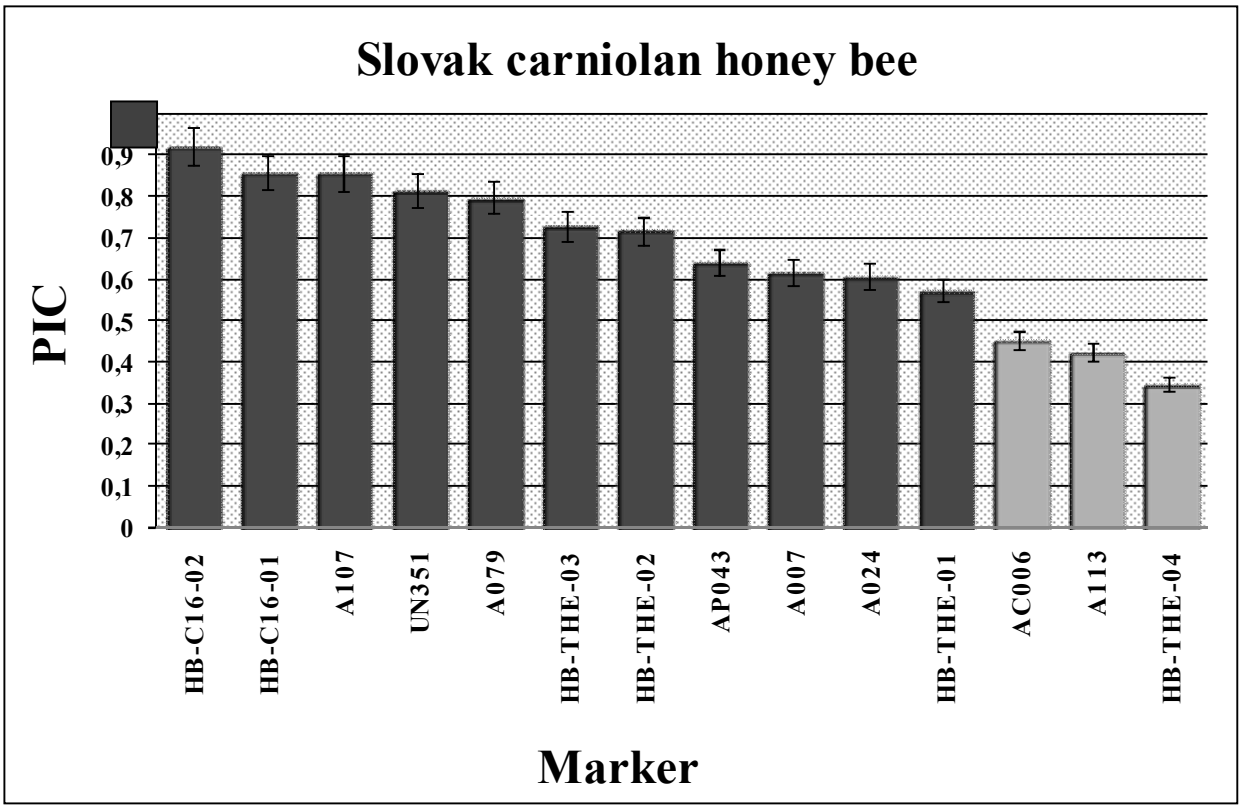

Figure 2 Values of PIC index for each microsatellite marker. Error bars with standard error are also shown

Legend: PIC ≥ 0,5

0,5 > PIC ≥ 0,25

On the basis of alleles frequency, the genotypes frequency and the detected number of heterozygotes we calculated the expected heterozygosity, the observed heterozygosity and Hardy-Weinberg equilibrium (Table 1).

The average value of expected heterozygosity H_E in tested set of individuals of the Slovak carniolan honey bee for all loci is 0,705 ±0, 15, the average value of observed heterozygosity H_O is 0,704±0, 18. On the basis of these results, it can be said that in the tested set of the

Slovak carniolan honey bees there is no significant loss of heterozygosity, for example due to inbreeding. From the total number of 11 markers we detected only in the case of 3 markers (HB-THE-02, AC006 a HB-THE-04) statistically significant deviation from the Hardy-Weinberg equilibrium (HWE). Genotype frequencies were in equilibrium for 11 markers.

Table 1 Values of expected heterozyosity (H_E), observed heterozygosity (H_O) and the state of Hardy-Weinberg equilibrium (HWE)

Marker	H_E	H_O	HWE
UN351	0,832	0,658	Yes
HB-THE-01	0,604	0,474	No
HB-THE-02	0,753	0,763	Yes
HB-THE-03	0,764	0,842	Yes
HB-THE-04	0,441	0,395	Yes
HB-C16-01	0,868	0,868	Yes
HB-C16-02	0,925	0,947	Yes
AC006	0,490	0,342	No
A079	0,820	0,868	Yes
AP043	0,693	0,700	Yes
A113	0,489	0,579	Yes
A024	0,673	0,789	Yes
A107	0,869	0,895	Yes
A007	0,651	0,737	Yes

We attempted to identify alleles specific for the Slovak carniolan honey bee breed. Such alleles could rise up the probability of correct determination of the Slovak carniolan honey bee breed in the future by genetic test, which could supplement classical morphometrical method (Dall´Olio et al., 2007). We identified allele H [202 bp] (HB-THE-03), alleles E [274 bp] and Q [300 bp] (HB-C16-01), alleles G [246 bp] and R [268 bp] (HB-C16-02), alleles A [148 bp] and D [158 bp] (A107) and alleles A [100 bp], F [110 bp], J [118 bp] (A007). However, the most of specific alleles occured with low frequency, therefore these results could be considered as a preliminary observation. It is necessary to further test more individuals of the Slovak carniolan honey bee and another breed, especially the Italian honey bee, import of which is highly probable in Slovakia, so it represents the highest risk of crossing with the Slovak carniolan honey bee.

CONCLUSION

Analyzing of 14 microsatellite markers on the set of 40 samples of the Slovak carniolan honey bee, we have identified a total of 123 alleles, with the mean value of 8,78 allele per locus. The highest PIC value (0,920) was observed for the marker HB-C16-02 and the lowest PIC value (0,344) was reached by the marker HB-THE-04, which had only 2 alleles. In summary, 11 of 14 tested markers are highly informative and constitute suitable set of markers for population studies and for the origin determination of the Slovak carniolan honey bee. The mean value of expected heterozygosity in the tested sample of honey bee was 0,705 ±0, 15 for all loci and the mean value of observed heterozygosity was 0,704±0, 18. In conclusion, there is no significant loss of heterozygosity in the tested set of the Slovak carniolan honey bees, for example due to inbreeding. Frequency of genotypes in the case of 11 microsatellite markers was in Hardy-Weinberg equilibrium.

Acknowledgments: This study was supported by the Slovak Research and Development Agency (APVV-0556-11) and the article was written during realization of the project „MARKERY No. 26220220033“supported by the Operational Programme Research and Development funded from the European Regional Development Fund.

REFERENCES

BOTSTEIN, D. – WHITE, R.L. – SKOLNICK, M – DAVIS, R. W. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. In *American Journal of Human Genetics*, vol. 32, 1980, p. 314-331.

BROOKER, R. J. Genetics : Analysis & Principles. New York : McGraw-Hill Companies, 761 p. ISBN 978-0-07-352528-0.

DALL´OLIO, R. – MARINO, A. – LODESANI, M. – MORITZ, R. F. A. Genetic characterization of Italian honeybee, Apis mellifera ligustica, based on microsatellite DNA polymorphism. In *Apidologie*, vol. 38, 2007, no. 2, p. 207-217.

ESTOUP, A. – GARNERY, L. – SOLIGNAC, M. – CORNUETT, J.-M. Microsatellite variation in honey bee (*Apis Mellifera* L.) populations : Hierarchical genetic structure and test of the infinite allele and stepwise mutation models. In *Genetics*, 1995, vol. 140, no. 2, p. 679-695.

CHLEBO, R. Plemenitba včely medonosnej. http://www.agroporadenstvo.sk/zv/vcely/vcely_02.htm (2012-02-09)

ESTOUP, A. – SOLIGNAC, M. – HARRY, M. – CORNUET, J.-M. Characterization of (GT), and (CT), microsatellites in two insect species: *Apis mellifera and Bombus terrestris.* In *Nucleic Acids Research*, vol. 21, 1993, no. 6, p. 1427-1431.

FAO. Draft guidelines on molecular genetic characterization of animal genetic resources. http://www.fao.org/docrep/meeting/022/am652e.pdf (2012-04-09).

LIU, K. – MUSE, S. V. PowerMarker : Integrated analysis environment for genetic marker data. In *Bioinformatics*, vol. 21, 2005, no. 9, p. 2128-2129.

NORSE, E. A. 1986. Conserving biological diversity in our national forests. Washington, D.C. : The Wilderness Society, 1986. ISBN 978-9990718768.

SHAIBI, T. – LATTORF, H. M. G. – MORITZ, R. F. A. A microsatellite DNA toolkit for studying population structure in *Apis mellifera*. In *Molecular Ecology Resources*, 2008, vol. 8, p. 1034-1036.

SHEPPARD, W. S. – McPHERON, B. A. Genetic variation in honey bees from an area of racial hybridization in western Czechoslovakia. In *Apidologie*, vol. 17, 1986, p. 21-32.

SOLIGNAC, M. – MOUGEL, F. – VAUTRIN, D. – MONNEROT, M. – CORNUET, J.-M. A third-generation microsatellite-based linkage map of the honey bee, *Apis mellifera*, and its comparison with the sequence-based physical map. In *Genome Biology*, vol. 8, 2007.

VAČKÁŘ, D. Ukazatele změn biodiverzity. Praha : Academia, 2005, 300 p. ISBN 80-200-1386-5.

VÚŽV – Národní referenční středisko uchování a využití genetických zdrojů hospodářských zvířat : Včela kraňská. http://www.genzdrojehz.wz.cz/set.htm (2012-01-27)

WILSON, E. O. Biodiversity. 1988. Washington, D.C.: National Academies Press, 1988. 521 p. ISBN 0-309-03783-2.