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Allozyme Variability in Populations of *A. mellifera mellifera* (Linnaeus 1758.), *A. m. carnica* (Pollman, 1879) and *A. m. caucasica* (Gorbachev, 1916*)* from Poland

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Abstract: The genetic variability in honey bee populations of three subspecies reared in Poland (*A. m. mellifera*, *A. m. carnica* and *A. m. caucasica*) has been studied by usage of allozymic analysis of six enzymic systems (MDH-1, ME, EST-3, ALP, PGM and HK) corresponding to 6 loci. All loci were found to be polymorphic in the populations studied. Two alleles were detected at ME locus, three alleles – at MHD-1, ALP, PGM and HK, and five alleles – at EST-3 loci. The most frequent and the private alleles were discussed as suitable genetic markers for the subspecies characterization. The observed and expected heterozygosities (H_o and H_e) ranged from 0.233 (*A. m. carnica*) to 0.311 (*A. m. mellifera*) and from 0.268 (*A. m. carnica*) to 0.327 (*A. m. caucasica*), respectively. Allele frequencies of all loci were used to estimate Nei's (1972) genetic distance, which was found to range from 0.025 (between *A. m. carnica* populatons) to 0.518 (between *A. m. caucasica* and *A. m. mellifera* populations). The estimated mean F_{ST} value from allozyme data was 0.28. UPGMA and Neighbour-Joining phylogenetic dendrograms were obtained by genetic distance matrix methods. The studied *A. m. mellifera*, *A. m. carnica* and *A. m. caucasica* populations were grouped in different clusters.

Key words: *Apis mellifera*, allozymes, genetic variability, Poland

Introduction

Honey bee, *Apis mellifera* L., has adapted to a wide variety of ecosystems and at the present moment about 26 subspecies and numerous ecotypes have been described, based upon behaviour, morphology, and molecular evidence. Europe is home to 10 of the 26 currently existing subspecies, including two of the most frequently used subspecies in commercial beekeeping worldwide (Ruttner 1988, Meixner *et al*. 2009). The biodiversity of honey bees of Europe and the conservation status of European subspecies have been recently reviewed in detail by De la Rúa *et al*. (2009).

The original range of the black bee *A. mellifera mellifera* extends from the Alps and Carpathians to the latitude of 60°N, from the Atlantic seashore of Western Europe eastward to the Urals but today the distribution of this subspecies is much reduced. In many Central and Northern European countries (such as Germany, Austria, Denmark and Sweden) these bees have hybridised much with *A. m. ligustica* and *A. m. carnica*. The extensive populations of this subspecies still exist in Great Britain, France, Switzerland and Poland (Meixner *et al*. 2009).

The original range of 'Carniolan bee' *A. m. carnica* extends across Central and Eastern Europe. This subspecies is spread on the territory of Austria, Slovenia, Croatia, Bosnia-Herzegovina, Albania, Serbia, Hungary and Romania. Due to its commer-

cially attractive traits such as high honey production and gentleness, *A. m. carnica* is now distributed almost worldwide.

Grey Caucasian honey bee *A. m. caucasica* originally occurs in Caucasus Mountains and could be found on the area covering former Soviet Union countries. It has been intensively used by beekeepers for more than 100 years (Ruttner 1988). The natural range of *A. m. caucasica* has been artificially expanded from Caucasus to Western Turkey and Bulgaria (Ivanova *et al*. 2011), while a significant number of hives have been introduced into Russia, Ukraine, Germany, Poland and France (Gromisz 1978, Ruttner 1988). During the past this subspecies was included in many selection programmes and was hybridized much with *A. m. carnica* and *A. m. macedonica*.

In Poland, honey bee breeding has been regulated by governmental low for over 30 years. Three subspecies – *Apis mellifera mellifera*, *Apis mellifera carnica* and *Apis mellifera caucasica* have been reared in the country.

The territory of Poland is a natural habitat of *Apis mellifera mellifera* (commonly called national, local or black bee). During the last century Polish beekeepers started importation of Carniolan queens, mainly from Danubian countries and Austria. At the same time *A. m. caucasica* bees were imported from Caucasus and from countries of former Soviet Union. The imported subspecies of bees, especially the Carniolan bees, began to dominate. At present time *A. m. carnica* occurs naturally in Southern Poland. In addition, there are many lines of *A. m. carnica* bees that were imported from Austria, Germany, Hungary and former Yugoslavia (Bieńkowska *et al*. 2008), that have been bred for improved performance. These lines are bred in different regions of Poland with a view to weather and flow conditions.

In order to protect the gene pool of the national subspecies *A. m. mellifera* closed bee breeding regions were created in the country.

Till the moment mainly clasical morphometry has been used to define the subspecies in Poland (Gromisz, Bornus 1971, Gromisz 1978, 1981, Kauhausenkeller 1991, Tofilski 2004, 2005, Rostecki *et al*. 2007, Gerula *et al*. 2009a, b). Recently, on the bases of allozyme analysis, three lines of *A. m. carnica* and *A. m. caucasica* from Poland and two lines of local Bulgarian *A. m. macedonica* honey bee were compared (Ivanova *et al*. 2011).

The purpose of the present research was to investigate, characterize and compare genetic variability in different populations of *A. m. mellifera, A. m. carnica* and *A. m. caucasica* from Poland and to give information about allele frequencies, levels of polymorphism and heterozygosity which could be used for needs of honey bee selection and conservation in the country.

Materials and Methods

Honey bee samples

Honey bee samples were collected from managed colonies of three subspecies (*A. m. mellifera*, *A. m. carnica* and *A. m. caucasica*) reared in Poland. Two populations of *A. m. carnica* (Pulawy and Olsztyn, marked as 1 and 2, respectively) were compared with two populations of *A. m. mellifera* (Augustovska) and *A. m. caucasica* (Pulawy), all of them reared in Poland. Totally 1121 worker bees were tested. Ten colonies per populations were included in this investigation.

The thorax homogenization and electrophoresis in polyacrylamide gel were performed according to Ivanova (1996).

Allozyme analysis

Six enzymic systems corresponding to six loci were studied: MDH (malate dehydrogenase, EC 1.1.1.37); ME (malic enzyme, EC 1.1.1.40); EST (esterase, EC 3.1.1), ALP (alkaline phosphatase, EC 3.1.3.1); PGM (phosphoglucomutase, EC 5.4.2.2) and HK (hexokinase, EC 2.7.1.1). Buffers and electrophoretic conditions for each enzymic system used were as in Boyer (1961), Gahne (1967), Shaw, Prasad (1970) and Ivanova (1996). Enzyme activities were visualised by histochemical staining (Harris, Hopkinson 1976).

Statistical Analyses

Allele frequencies, mean number of alleles per locus, proportion of polymorphic loci at the 95% level, observed (H_o) and expected (H_e) heterozygosity, deviation from the Hardy-Weinberg equilibrium and Nei's genetic distance (D) (Nei 1972), were calculated using BIOSYS-1 (Swofford, Selander 1981). Phylogenetic trees were constructed using Nei's (1972) genetic distance, by UPGMA (Sneath, Sokal 1973) and neighbour-joining (Saitou, Nei 1987) methods using the PHYLIP (Felsenstein 1993) software package.

Results

In this research the enzyme systems studied (MDH-1, ME, EST-3, ALP, PGM and HK) were polymorphic in all of the populations, at the 95% level, having two to four different alleles in the studied populations (Fig. 1-6, Table 1). In total, two alleles were detected at ME (ME^{100} and ME^{106}), three alleles – at MDH-1 (MDH^{65}, MDH^{80} and MDH^{100}), ALP (ALP^{80}, ALP^{90} and ALP^{100}), PGM (PGM^{80}, PGM^{100} and PGM^{114}) and HK (HK^{87}, HK^{100} and HK^{110}) loci and five alleles – at EST-3 (EST^{88}, EST^{94}, EST^{100}, EST^{105} and EST^{118}) locus.

In the present study, the mean number of alleles per locus varied from 2.3 (*A. m. carnica* from Pulawy) to 2.7 (*A. m. caucasica*). The estimated percentage of polymorphic loci was 100% in *A. m. caucasica* population, 83.3% in *A. m. carnica* from Olsztyn and 66.7% in others, using the 0.95 criterion.

The observed and expected heterozygosities (H_o and H_e) ranged from 0.233 (*A. m.* from Pulawy) to 0.311 (*A. m. mellifera*) and from 0.268 (*A. m. carnica* from Olsztyn) to 0.327 (*A. m. caucasica*), respectively (Table 1).

There were significant deviations of genotype frequencies from Hardy-Weinberg expectations at most of the loci in most populations ($P \geq 0.001$). Chi-Square tests showed that the deviations were generally in favor of homozygotes.

The estimated mean F_{ST} value was 0.280 which shows that 28% of the overall genetic diversity observed was among populations, as opposed to 72% within populations.

The values of genetic distance (Nei 1972) were calculated using the allele frequencies and ranged from 0.025 (between both *A. m. carnica* populations) to 0.518 (between *A. m. caucasica* and *A. m. mellifera* populations) – Table 2.

In both, UPGMA and Neighbor-Joining dendrograms, *A. m. carnica, A. m. mellifera* and *A. m. caucasica* are clustered separately (Fig. 7).

Discussion

All enzyme loci studied here were found to be polymorphic. In similar studies, in total five alleles on MDH-1 locus were detected (Gardside 1980, Nunamaker *et al*. 1984, Badino *et al*. 1983, Badino *et al*. 1985, Badino *et al*. 1988, Sheppard 1988, Sheppard, Berlocher 1984, 1985, Sheppard, McPheron 1986, Lobo *et al*. 1989, Meixner *et al*. 1994, Kandemir, Kence 1995, Dedej *et al*. 1996, Kandemir *et al*. 2000, Bouga *et al*. 2005, Ivanova 2010) in different populations from Europe, Brazil and USA. In the present research we observed three alleles at this locus in all of studied Polish populations of *A. m. carnica*, *A. m. mellifera* and *A. m. caucasica*. MDH^{100} was the most common allele in *A. m. carnica* (with frequency 0.52 and 0.789 – for Pulawy and Olsztyn, respectively) and *A. m. caucasica* (0.551) populations. MDH^{80} was found to be with the highest frequency in *A. m. mellifera* (0.576) population, while its frequency was lower in *A. m. caucasica* – 0.214 and *A. m. carnica* from Olsztyn and Pulawy (0.132 and 0.08, respectively), Fig. 1.

Three alleles were found at ME locus (ME^{70}, ME^{100} and ME^{106}) in *A. mellifera* populations in Norway (Sheppard, Berlocher 1984), Italy (Sheppard, Berlocher 1985) and Western Czechoslovakia (Sheppard, McPheron 1986). ME locus was found to be invariant in investigations of Kandemir *et al*. (2000), Kandemir *et al*. (2005) and Dedej *et al*. (1996), but according to Bouga *et al*. (2005) this locus is polymorphic with two alleles – ME^{100} and ME^{79} in *A. m. macedonica* populations from Greece. ME^{100} was found to be fixed in *A. m. carnica* populations from Serbia (Ivanova *et al*. 2010). Ivanova *et al*. (2011) reported two alleles (ME^{100} and ME^{106}) in selectively reared lines of *A. m. carnica* and *A. m. caucasica* from one of the selection bases in Poland. In our present research the same two alleles of ME locus were found in the studied population. The ME^{100}

Table 1. Percent of polymorphic loci, Observed (Ho) and Expected (He) Heterozygosity values in the populations studied.

Population	Mean no. of alleles per locus (± s.e)	Percent Polymorphic loci (P=0.95)	H_o	H_e
A. m. carnica 1	2.3±0.2	66.7	0.233±0.095	0.271±0.109
A. m. carnica 2	2.7±0.3	83.3	0.234±0.054	0.268±0.084
A. m. mellifera	2.5±0.2	66.7	0.311±0.139	0.272±0.104
A. m. caucasica	2.7±0.2	100	0.238±0.109	0.327±0.1

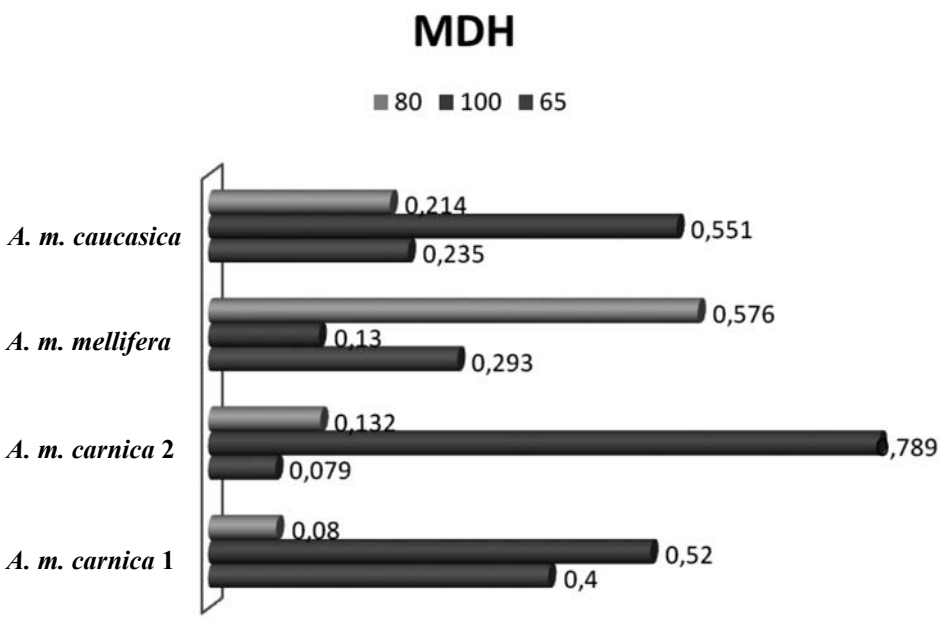

Fig. 1. MDH polymorphism and MDH allele frequencies in the studied populations.

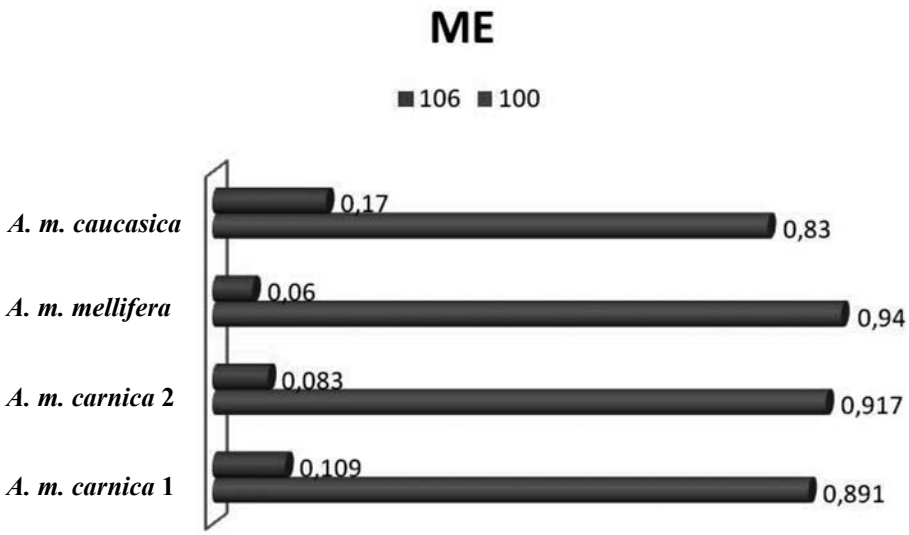

Fig. 2. ME polymorphism and ME allele frequencies in the studied populations.

allele was with the highest frequency in *A. m. mellifera* (0.94). In this study ME[106] was with the highest frequency in '*caucasica'* bees (0.170). In the studied '*carnica'* populations this allele was with frequencies 0.109 and 0.083 (for Pulawy and Olsztyn populations, respectively), Fig. 2.

EST-3 locus was polymorphic and exhibited three alleles in Czechoslovakian (Sheppard, McPheron 1986), central Anatolian (Kandemir, Kence 1995), Greek (Bouga *et al*. 2005) honey bees and also in some populations from Bulgaria, Serbia, Montenegro and Greece (Ivanova 2010; Ivanova *et al*. 2010). In the present investigation, totally five alleles were detected in the studied populations*:* two – in '*carnica'* bees from Pulawy (EST[94] and EST[100]); four – in '*carnica*' bees from Olsztyn (EST[88]**,** EST[94], EST[100] and EST[105]); two – for '*mellifera'* bees (EST[100] and EST[105]) and three – for '*caucasica'* bees (EST[94], EST[100] and EST[118]), Fig. 3.

The ALP locus was found to be polymorphic with two alleles, ALP[100] and ALP[80]. ALP[80] was more frequent allele in Greece (Bouga *et al*. 2005) and in Bulgaria (Ivanova *et al*. 2010). In the present research ALP locus had three alleles in all of the studied honey bee populations. In the investigated here *A. m. mellifera* population, the most frequent allele

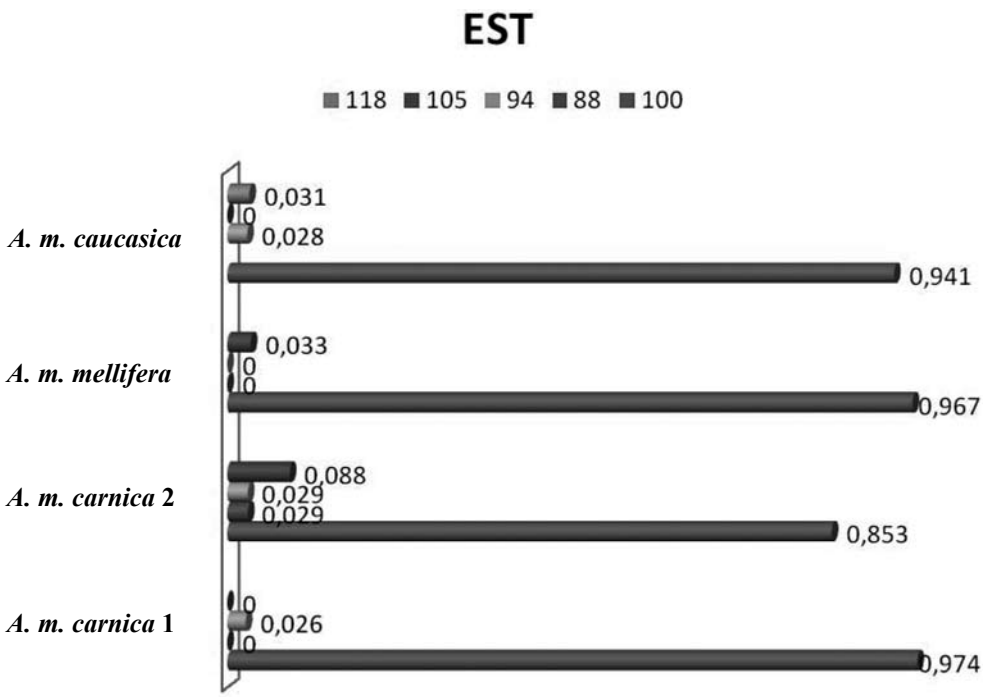

Fig. 3. EST polymorphism and EST allele frequencies in the studied populations.

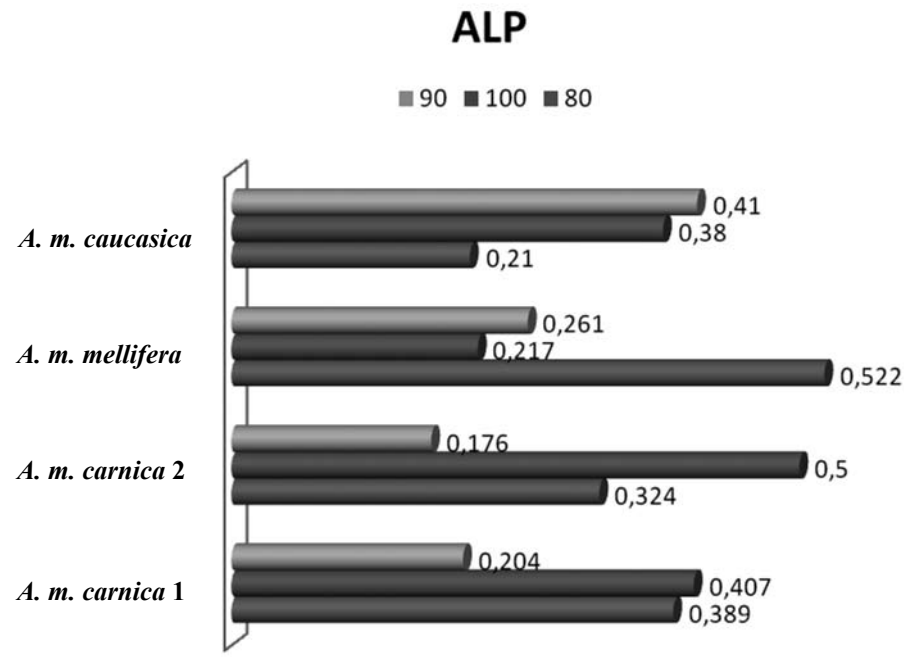

Fig. 4. ALP polymorphism and ALP allele frequencies in the studied populations.

was ALP^{80} (0.522), while in *A. m. caucasica* populations the most frequent allele was ALP^{90} (0.41) and in *A. m. carnica* – it was ALP^{100} allele), Fig. 4.

The PGM locus was found to be invariant in studies of Mestriner, Contel (1972), Brueckner (1974), Nunamaker, Wilson (1980), Badino *et al*. (1983), Sheppard, Berlocher (1985). Del Lama *et al*. (1985) first reported the presence of three alleles at this locus in Africanized bee populations and two alleles in *A. m. carnica* originating from Germany. Meixner *et al.* (1994) found three alleles of which PGM^{120} was previously unreported. PGM locus was found to be polymorphic with two alleles (PGM^{100} and PGM^{114}) in populations from Serbia, Montenegro, Bulgaria and Greece (Ivanova *et al*. 2010). In the present study a third allele (PGM^{80}) was found in *A.*

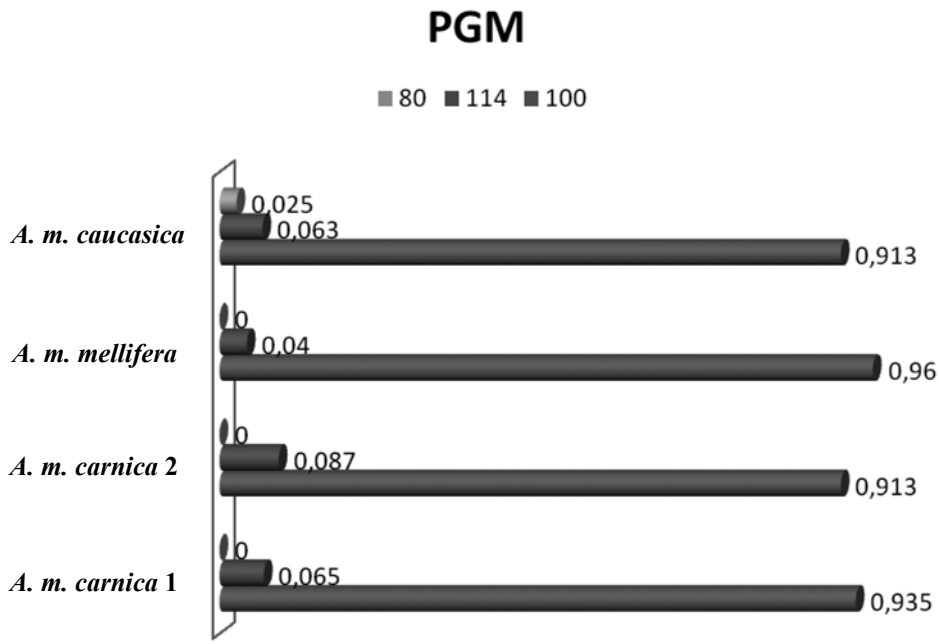

Fig. 5. PGM polymorphism and PGM allele frequencies in the studied populations.

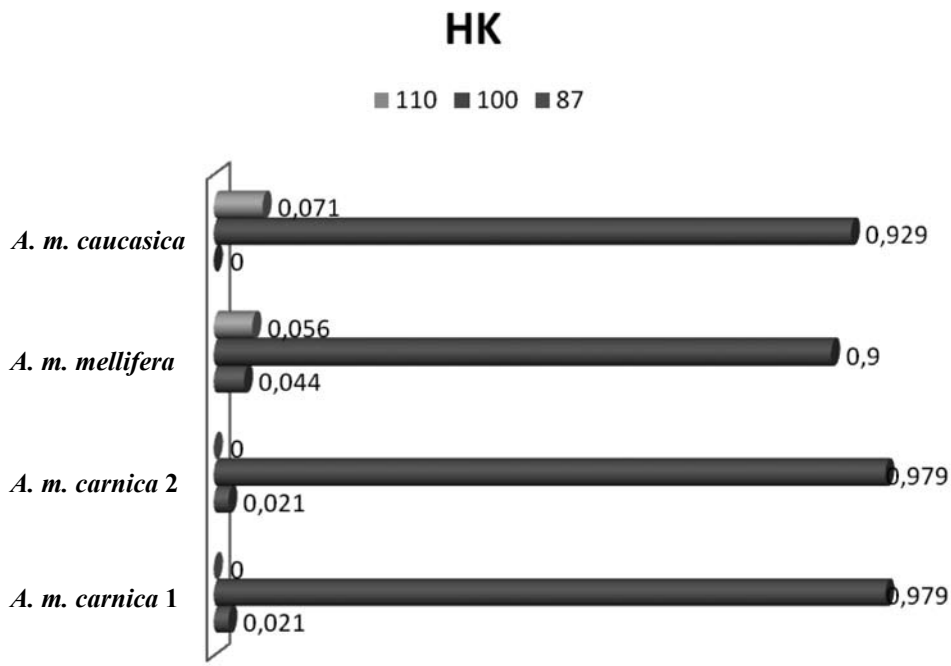

Fig. 6. HK polymorphism and HK allele frequencies in the studied populations.

m. caucasica population from Poland. Its frequency was calculated as 0.025, Fig. 5.

HK locus was monomorphic in Norwegian, Italian (Sheppard, Berlocher 1985), Czechoslovakian (Sheppard, McPheron 1986), Greek (Badino *et al.* 1988) and German (Del Lama *et al.*, 1990) honey bee populations. It was found to be polymorphic with two to five alleles in studies of Del Lama *et al.* (1988, 1990), Kandemir, Kence (1995), Kandemir *et al.* (2000). Ivanova *et al*. (2010) described one more allele – HK^{121} in Serbian *A. m. carnica* populations. Totally, three alleles were found at HK locus (HK^{87}, HK^{100} and HK^{110}) in the present investigation. HK^{100} was more common in all of the studied populations. In *A. m. carnica* populations HK^{87} allelic variant was detected, in *A. m. caucasica* – HK^{110},

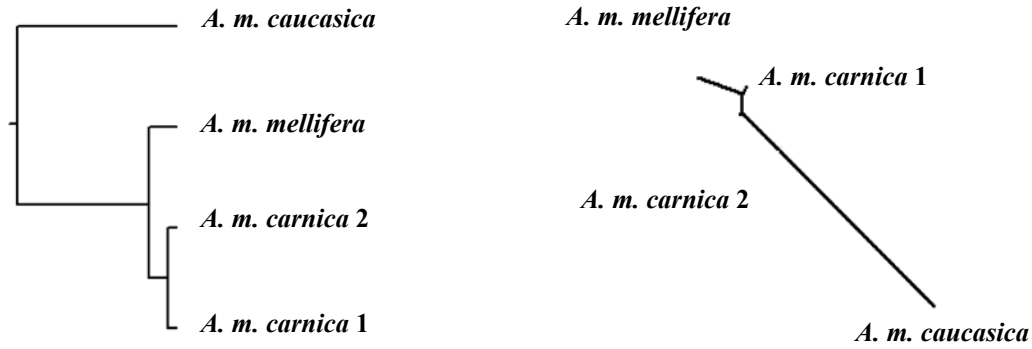

Fig. 7. Relationships between populations as shown in UPGMA (a) and Neighbour-Joining (b) phylogenetic dendrograms.

Table 2. Nei's Genetic distances.

Population	*A. m. carnica* 1	*A. m. carnica* 2	*A. m. mellifera*	*A. m. caucasica*
*A. m. carnica*1	***	0.025	0.057	0.424
A. m. carnica 2		***	0.1	0.417
A. m. mellifera			***	0.518
A. m. caucasica				***

and in *A. m. mellifera* – HK^{87} and HK^{110}, Fig. 6**.**

Data about the polymorphism found in the present study, are in agreement with some of the results reported previously by Ivanova *et al*. (2011) concerning the studied *A. m. carnica* and *A. m. caucasica* honey bee lines.

High percentage of polymorphic loci (66.7% – 100%) and moderate value (mean F_{ST} = 0.28) of genetic differentiation among the studied populations were detected.

In UPGMA and Neighbour-Joining phylogenetic dendrograms (Fig. 7) the investigated *A. m. mellifera*, *A. m. carnica* and *A. m. caucasica* populations were grouped in three different clusters which demonstrated clear genetic differentiation between them.

The results of this research provide new comparative information concerning the genetic variability in different *A. m. mellifera, A. m. carnica* and *A. m. caucasica* honey bee populations from Poland. Data about allozyme variability in *A. m. mellifera* populations and their comparison with different *A. m. carnica* and *A. m. caucasica* populations from Poland are reported and discussed here for first time. The alleles with the highest frequency, the private alleles and the genetic differences found between the studied populations could be successfully used as genetic markers in further population-genetic investigation in Poland and in Europe, in order to determine conservation areas of local and national interests.

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