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Additional Information on Allozyme Variability of Honey Bees, *Apis mellifera* Linnaeus, 1758, from Bulgaria

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Abstract: The genetic variability of honey bee populations from 24 Bulgarian provinces and populations of *Apis mellifera carnica*, *A. m. caucasia*, *A. m. ligustica* and *A. m. macedonica* were studied. I used allozymic analysis of six enzymic systems (MDH, ME, EST, ALP, PGM and HK) corresponding to 6 loci. All of studied loci were found to be polymorphic in most of the populations. Four alleles were detected at Mdh-1, Me and Pgm loci and three alleles at Alp and Hk loci. Est-3 locus was found to be polymorphic with six alleles. In the constructed phylogenetic trees, the subspecies of *A. mellifera* were clustered separately. All Bulgarian populations were grouped together with *A. m. macedonica*, forming the second branch in this cluster. The results confirm the existence mainly of only one subspecies, *A. m. macedonica*, on the territory of Bulgaria. This paper provides and discusses for first time detailed information concerning the genetic variability and differentiation among Bulgarian populations of honey bee throughout the country. My findings could be useful for conservation and selection purposes.

Key words: *Apis mellifera*, allozymes, polymorphism, Bulgaria, phylogenetics

Introduction

About 26 subspecies of *A. mellifera* Linnaeus, 1758 are recognised (Ruttner 1988, 1992, Sheppard *et al*. 1997, Sheppard, Meixner 2003, Meixner *et al*. 2009), mainly on the basis of classical morphometry. According to Ruttner's (1988) morphometric analysis, the subspecies *A. m. macedonica* Ruttner, 1988 occurs in Bulgaria. In contrast, according to Petrov (1990), a native type (named A. m. *rodopica*) exists in the country. Until 1980, the local Bulgarian bee was threatened by many activities, including queen breeding and importation of foreign queens of *A. m. ligustica* Spinola, 1806, *A. m. carnica* Pollmann, 1879 and *A. m. caucasia* Pollmann, 1889 that modify local bees through hybridisation. Since 1999, an updated national programme for bee breeding is enforced in Bulgaria, with conservation of the local Bulgarian honey bee gene pool being its main purpose. In order to determine the race standard of Bulgarian honey bees, a morpho-ethological analysis by specific characteristics has been carried out (Petrov 1990, 1995, 1996, 2000). Biochemical-genetic studies on polymorphism in isoenzyme systems in Bulgarian populations have also been carried out (Ivanova *et al.* 2007, 2010a, Ivanova, Bouga 2009, Ivanova *et al*. 2012, Francis *et al*. 2014). In the last years, in order to characterise the polymorphism in different populations of local honey bee which is part of the European genetic resource of *Apis mellifera*, it has been purposefully worked for applying simultaneously different approaches, i.e. morphometrical, ethological, isoenzymical and DNA analysis (Ivanova, Bouga 2009, Ivanova *et al*. 2010b),.

Although there are different studies concerning race status and the degree of genetic diversity of Bulgarian honey bee, they are mainly based on classical morphometry with some comparison between selectively reared in Bulgarian lines (Ivanova *et al*. 2011). Genetic structure of honey bee populations from the whole country and populations of *A. m. macedonica*, *A. m. carnica, A. m. ligustica* and *A. m. caucasia* still have not been compared. The aim of this study is to examine: 1) allozyme variation in honey bee popu-

lations from different locations of all geographical regions of the country; 2) phylogenetic relationships between Bulgarian honey bee populations and *A. m. macedonica*, *A. m. carnica, A. m. ligustica* and *A. m. caucasia* populations using allozyme analysis.

Materials and Methods

Honey bee populations from 24 provinces and more than 100 locations from Bulgaria and populations recognized according to morphometric analysis (Ruttner 1988) as *A. m. carnica* (originated from Germany), *A. m. caucasia* (originated from Poland), *A. m. ligustica* (originated from Italy) and *A. m. macedonica* (originated from Greece) were studied. In total, about 5000 worker bees (five colonies per population, seven to 12 individuals per colony) were tested. The thorax homogenisation and electrophoresis in 7.5% polyacrylamide gel were done according to Ivanova (1996). Six enzymic systems 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 according to Ivanova (1996) and Ivanova *et al*. (2010b). Enzyme activities were visualised using histochemical staining (Harris, Hopkinson 1976) and allozymes were numbered according to their relative anodal mobility.

The alloenzymic results were statistically analysed using BIOSYS-1 (Swofford, Selander 1981) and PHYLIP (Felsenstein 1993) software packages. UPGMA (Sneath, Sokal 1973) and neighbour-joining (Saitou, Nei 1987) phylogenetic trees were obtained using genetic distance matrix methods.

Results

I found that all the studied enzyme systems were polymorphic in most of the populations, having three to six different alleles (Table 1). In total, for Bulgarian honey bee populations, three alleles were detected at Mdh-1 (MDH^{65}, MDH^{80} and MDH^{100}), four at Me locus (ME^{90}, ME^{100}, ME^{106} and ME^{115}), six at Est-3 locus (EST^{80}, EST^{88}, EST^{94}, EST^{100}, EST^{105} and EST^{118}), three at Alp (ALP^{80}, $ALP^{\mathbf{90}}$ and $ALP^{\mathbf{100}}$), four at Pgm (PGM^{80}, PGM^{100}, PGM^{114} and PGM^{125}) and three at Hk (HK^{87}, HK^{100} and HK^{110}) locus. One more allele of Mdh-1 locus (MDH^{125}) was found in *A. m. carnica* population. While ME^{100} allele was fixed in *A. m. carnica*, ME^{90} was absent. ME^{106} was present in *A. m. ligustica*. Me^{115} was found to be present only in one Bulgarian population. EST^{80}, $PGM^{125}$and HK^{87} alleles were not detected in populations of *A. m. carnica*, *A. m. caucasia*, *A. m. ligustica* and *A. m. macedonica.* EST^{88} was found only in *A. m. macedonica*, EST^{94} in *A. m. macedonica* and *A. m. caucasia*, EST^{105} – in *A. m. carnica* and *A. m. ligustica*, EST^{118} in *A. m. carnica* and *A. m. caucasia*. $ALP^{\mathbf{90}}$ allele was absent from the populations of *A. m. carnica* and *A. m. caucasia*. PGM^{100} was fixed in "*carnica*" population and PGM^{80} was present only in "ligustica" population (Table 1).

I found that the mean number of alleles per locus in Bulgarian populations varied between 1.8 (Razgrad) and 3.5 (Burgas). The calculated percentage of polymorphic loci ranged between 50% (in seven of the studied populations) and 100% (in only one of the studied provinces), using the 0.95 criterion. The observed and expected heterozygosities (H_o and H_e) for the Bulgarian populations ranged from 0.142 (Vidin) to 0.253 (Sliven) and from 0.219 (Razgrad and Ruse) to 0.296 (Montana), respectively (Table 2). At the same time, the calculated percentage of polymorphic loci in other studied subspecies was as follows: 66.7% in *A. m. carnica* and *A. m. ligustica* populations; 83.3% in *A. m. macedonica* and 100% in *A. m. caucasia* populations. For these populations the observed and expected heterozygosities (H_o and H_e) ranged from 0.163 (*A. m. carnica*) to 0.246 (*A. m. ligustica*) and from 0.248 (*A. m. macedonica*) to 0.326 (*A. m. caucasia*), respectively (Table 2).

The estimated mean F_{ST} value for all studied populations was 0.0256 which showed that 2.56% of the overall genetic diversity observed was among populations, as opposed to 97.44% within populations.

Allele frequencies of all loci were used to estimate Nei's (1972) genetic distance, which was found to range between 0.001 and 0.078.

In UPGMA and neighbour-joining phylogenetic trees (Figs. 1 and 2), *A. m. caucasia*, *A. m. carnica*, *A. m. ligustica* and *A. m. macedonica* were clustered separately. All Bulgarian populations were grouped together with *A. m. macedonica* forming the second branch in this cluster.

Discussion

The results of the present study show some differences between Bulgarian and *A. m. macedonica* honey bee populations in regards to polymorphism found at almost all loci (Table 1).

Concerning Mdh-1 locus, five alleles were found in analogous studies (Meixner *et al*. 1994, Kandemir *et al*. 2000, Ivanova *et al*. 2010b) and three alleles in honey bee populations from Greece (Bouga *et al*. 2005). In a study by Dedej *et al*. (1996)

Table 1. Allele frequencies in populations studied

Locus	Allele	Lig	Cau	Car	Mac	North East					North Central				North West		South West				South Central						South East		
						Vrn	Dbr	Shm	Rzg	Trg	Rus	V.Tr	Plv	Lov	Vdn	Mon	Blg	Knd	Prn	Sf	Sm	Kr	Has	Pz	Pld	St.Z	Brg	Yam	Sl
MDH1	**65**	0.553	0.235	0.154	0.13	0.467	0.353	0.435	0.455	0.483	0.461	0.451	0.563	0.491	0.304	0.333	0.444	0.505	0.384	0.487	0.407	0.462	0.441	0.494	0.4	0.641	0.398	0.443	0.481
	100	0.426	0.551	0.577	0.685	0.533	0.647	0.565	0.545	0.477	0.539	0.549	0.437	0.506	0.696	0.464	0.556	0.495	0.616	0.513	0.587	0.538	0.537	0.506	0.6	0.359	0.596	0.557	0.519
	80	0.021	0.214	0.115	0.185	0	0	0	0	0.04	0	0	0	0.003	0	0.202	0	0	0	0	0.006	0	0.023	0	0	0	0.006	0	0
	125	0	0	0.154	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ME	100	0.826	0.826	1	0.929	0.958	0.939	0.979	0.9	0.877	0.951	0.853	0.947	0.955	0.864	0.884	0.915	0.869	0.951	0.833	0.899	0.943	0.903	0.963	0.901	0.891	0.91	0.931	0.949
	106	0	0.174	0	0.014	0.042	0.061	0.021	0.1	0.085	0.049	0.118	0.045	0.017	0.136	0.116	0.071	0.03	0.033	0	0.074	0.041	0.097	0.037	0.086	0.087	0.038	0.025	0.026
	90	0.174	0	0	0.057	0	0	0	0	0.038	0	0.029	0.008	0.028	0	0	0.005	0.101	0.016	0.167	0.027	0.016	0	0	0.013	0.022	0.052	0.044	0.026
	115	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.009	0	0	0	0	0	0	0	0	0	0	0	0
EST3	**80**	0	0	0	0	0.022	0.052	0.036	0	0	0	0	0.026	0.023	0	0	0.008	0	0.021	0	0	0	0.021	0.116	0.008	0	0.017	0	0.015
	100	0.955	0.944	0.813	0.891	0.955	0.94	0.893	1	0.971	0.993	0.931	0.967	0.938	1	0.924	0.966	0.989	0.979	0.975	0.942	0.898	0.89	0.884	0.958	0.957	0.943	0.959	0.963
	88	0	0	0	0.022	0.022	0.007	0	0	0	0	0	0	0	0	0.043	0	0	0	0	0	0	0	0	0	0	0.008	0	0
	118	0	0.028	0.063	0	0	0	0.071	0	0.007	0.007	0.014	0.007	0.008	0	0.033	0.025	0	0	0.025	0.042	0.018	0.031	0	0.02	0.043	0.009	0	0
	94	0	0.028	0	0.087	0	0	0	0	0.022	0	0	0	0	0	0	0	0.011	0	0	0	0.084	0.059	0	0.006	0	0.013	0.01	0.022
	105	0.045	0	0.125	0	0	0	0	0	0	0	0.056	0	0.031	0	0	0	0	0	0	0.017	0	0	0	0.008	0	0.009	0.031	0
ALP	80	0.778	0.240	0.111	0.524	0.518	0.46	0.568	0.625	0.555	0.619	0.537	0.589	0.587	0.526	0.552	0.511	0.539	0.508	0.523	0.559	0.337	0.467	0.638	0.507	0.478	0.375	0.5	0.409
	100	0.222	0.380	0.722	0.476	0.482	0.5	0.341	0.375	0.375	0.381	0.435	0.411	0.41	0.474	0.448	0.467	0.461	0.492	0.477	0.441	0.663	0.489	0.362	0.46	0.522	0.452	0.458	0.523
	90	0	0.380	0.167	0	0	0.04	0.091	0	0.07	0	0.028	0	0.003	0	0	0.022	0	0	0	0	0	0.044	0	0.033	0	0.173	0.042	0.068
PGM	100	0.870	0.923	1	0.981	0.872	0.949	0.979	0.955	0.978	0.917	0.899	0.935	0.914	0.935	0.913	0.963	0.957	0.921	0.942	0.918	0.951	0.955	0.892	0.924	0.891	0.93	0.918	0.836
	114	0.130	0.058	0	0.019	0.128	0.051	0.021	0.045	0.022	0.071	0.101	0.065	0.086	0.065	0.087	0.033	0.032	0.053	0.058	0.082	0.049	0.036	0.108	0.076	0.109	0.068	0.082	0.145
	80	0.019	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.002	0	0
	125	0	0	0	0	0	0	0	0	0	0.012	0	0	0	0	0	0.004	0.011	0.026	0	0	0	0.009	0	0	0	0	0	0.018
HK	**87**	0	0	0	0	0.02	0.033	0.017	0.034	0.018	0.015	0.037	0.014	0.018	0.013	0.021	0.007	0.013	0	0	0.006	0.026	0.022	0.023	0.06	0.03	0.014	0.008	0
	100	0.981	0.929	0.915	0.943	0.944	0.951	0.966	0.966	0.976	0.961	0.951	0.949	0.974	0.975	0.938	0.978	0.97	0.959	0.967	0.974	0.974	0.973	0.974	0.927	0.97	0.966	0.963	0.972
	110	0.019	0.019	0.085	0.057	0.035	0.016	0.017	0	0.006	0.024	0.011	0.037	0.008	0.013	0.042	0.015	0.017	0.041	0.033	0.019	0	0.004	0.003	0.013	0	0.02	0.028	0.028

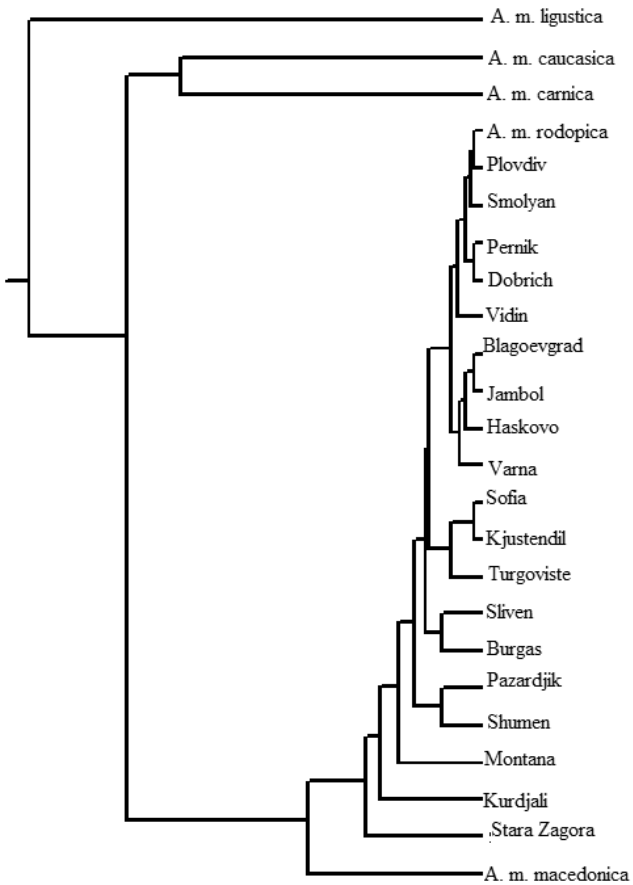

Fig. 1. UPGMA dendrogram (Sneath, Sokal 1973) demonstrating the relationships between the studied populations

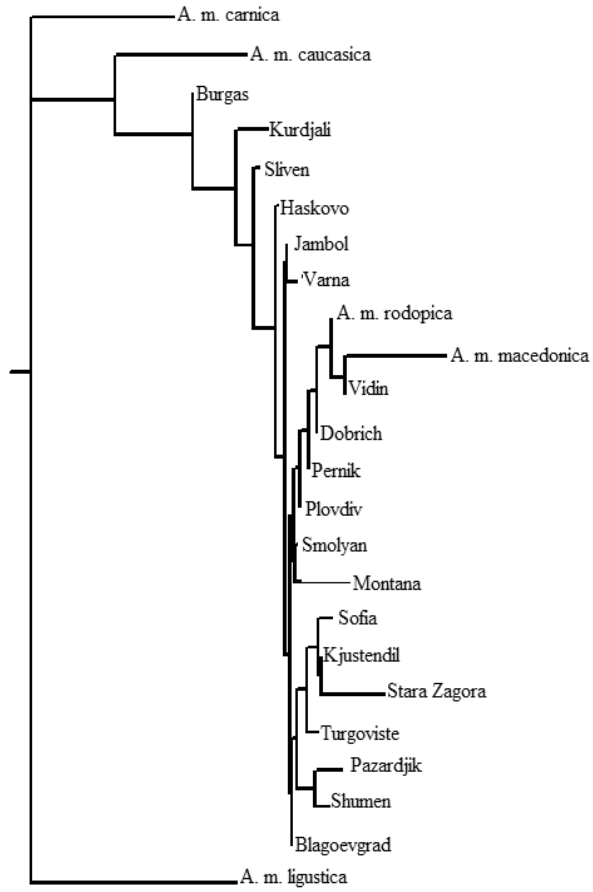

Fig. 2. Neighbour-joining dendrogram (Saitou, Nei, 1987) demonstrating the relationships of the studied populations

for *A. m. macedonica*, only two alleles were found at Mdh-1 locus. In the present study, it is very interesting to note the detection of MDH-1^{125} allele only in *A. m. carnica* population (0.154).

Concerning Me locus, three alleles (ME^{90}, ME^{100} and ME^{106}**)** were found in *A. mellifera* populations from Norway, Italy and Western Czechoslovakia (Sheppard, Berlocher 1984, 1985, Sheppard, McPheron 1986). Dedej *et al*. (1996) reported no polymorphism at the Me locus but, according to Bouga *et al*. (2005), this locus is polymorphic with two alleles in *A. m. macedonica* populations from Greece. In the present study, the Me^{100} and Me^{106} alleles were found in all Bulgarian populations, in *A. m. macedonica* and *A. m. caucasia* populations, Me^{90} was observed in most of the Bulgarian populations, in *A. m. macedonica* and *A. m. ligustica*. The allele Me^{115} was detected only in one of the studied populations from Bulgaria (0.009).

Est-3 locus exhibited three alleles, EST^{70}, EST^{100} and EST^{130} in honey bee populations from former Czechoslovakia (Sheppard, McPheron 1986) and Central Anatolia (Kandemir, Kence 1995). Three alleles were detected also in *A. m. macedonica* from Greece (Bouga *et al.* 2005) but in this study six alleles were detected. The Est^{80} allele was found only in Bulgarian populations. EST^{88} was found only in *A. m. macedonica* and in some of Bulgarian populations.

Concerning the Alp, two alleles (ALP^{100} and ALP^{80}**)** were detected in populations from Greece (Bouga *et al*. 2005) and Bulgaria (Ivanova *et al*. 2010a). In the present research, a third allele ALP^{90} was found at this locus. All three alleles were detected as present in 11 of the Bulgarian populations and also in *A. m. carnica* and *A. m. caucasia* populations.

Del Lama *et al.* (1985) first reported the presence of three alleles at Pgm 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 Bulgaria (Ivanova *et al*. 2007, 2010). In this study, two more alleles (Pgm^{80} and Pgm^{125}) were found only in some of the Bulgarian populations.

HK locus was found as monomorphic in Norwegian, Italian, Czechoslovakian, Greek and German (Sheppard, Berlocher 1985, Sheppard, McPheron 1986, Badino *et al*. 1988, Del Lama *et*

Table 2. Mean no. of alleles per locus, Percent of polymorphic loci, Observed (Ho) and Expected (He) Heterozygosity values in the populations studied

Geographical Regions	Population	Mean no. of alleles per locus	Percent Polymorphic loci (P=0.95)	H_o	H_e
North East	Varna	2.3±0.2	66.7	0.244±0.089	0.251±0.082
	Dobrich	2.5±0.2	83.3	0.23±0.089	0.237±0.084
	Shumen	2.5±0.2	50	0.187±0.073	0.234±0.096
	Razgrad	1.8±0.2	50	0.223±0.1	0.219±0.087
	Turgoviste	2.8±0.2	50	0.205±0.103	0.243±0.099
North Central	Ruse	2.3±0.2	50	0.161±0.072	0.219±0.087
	V.Turnovo	2.7±0.2	83.3	0.229±0.081	0.282±0.076
	Pleven	2.5±0.2	83.3	0.162±0.07	0.228±0.083
	Lovech	3±0.3	66.7	0.181±0.065	0.235±0.084
North West	Vidin	2±0.3	66.7	0.142±0.052	0.224±0.084
	Montana	2.5±0.2	100	0.19±0.056	0.296±0.09
South West	Blagoevgrad	3±0.3	50	0.213±0.096	0.226±0.091
	Kjustendil	2.5±0.2	50	0.214±0.1	0.233±0.09
	Pernik	2.3±0.2	50	0.204±0.08	0.224±0.085
	Sofia	2±0	66.7	0.218±0.091	0.255±0.087
South Central	Smolyan	2.7±0.2	83.3	0.216±0.087	0.249±0.08
	Kurdjali	2.3±0.2	66.7	0.18±0.077	0.232±0.079
	Haskovo	3±0.3	66.7	0.202±0.079	0.264±0.088
	Pazardjik	2.2±0.2	66.7	0.18±0.066	0.247±0.079
	Plovdiv	3±0.4	83.3	0.24±0.111	0.259±0.08
	St.Zagora	2.2±0.2	66.7	0.221±0.077	0.252±0.078
South East	Burgas	3.5±0.5	83.3	0.206±0.084	0.265±0.095
	Jambol	2.7±0.2	66.7	0.223±0.093	0.245±0.088
	Sliven	2.7±0.2	66.7	0.253±0.089	0.262±0.092
Subspecies					
A.m.macedonica		2.5±0.2	83.3	0.166±0.088	0.248±0.083
A.m.carnica		2.3±0.5	66.7	0.163±0.59	0.263±0.105
A.m.caucasica		2.7±0.2	100	0.234±0.109	0.326±0.101
A.m.ligustica		2.2±0.2	66.7	0.246±0.87	0.254±0.72

al. 1990) honey bee populations. It was detected to be polymorphic with two alleles (HK^{87} and HK^{100}) in Africanized bee populations from Brazil and Central America (Del Lama *et al*. 1988, 1990). Later studies determined four alleles at this locus (Kandemir, Kence 1995). Kandemir *et al*. (2000) detected one more allele (HK^{77}) in honey bee populations from Turkey. In the present study three alleles were found and the HK^{87} allele was present only in Bulgarian populations.

This investigation presents a comparative allozyme analysis of the indigenous population of *A. mellifera* in Bulgaria. The population structure in its native range, including previously unstudied areas was considered and characterised based on allozyme polymorphism of six loci. The results show that the honey bee populations included in the present study correspond to the subspecies *A. m. macedonica* as it was described by Ruttner (1988) based on classical morphometry. These populations are genetically clearly distinct from *A. m. carnica*, *A. m. ligustica* and *A. m. caucasia*. They confirm the existence mainly of only one subspecies, *A. m. macedonica*, on the territory of the country.

As it was shown in both of the phylogenetic trees (Figs. 1 and 2), a clear subdivision between *A. m. macedonica* populations from Bulgaria and Greece became visible. All of the studied honey bee populations from Bulgaria were clustered separately from the *A. m. macedonica* from Greece. This suggests that the local Bulgarian honey bee named by Petrov (1990) as "*rodopica*" could be a different ecotype of *A. m. macedonica*, although no indication of geographical variations within the *A. m. macedonica* subspecies was mentioned by Ruttner (1988).

The results of this study are in agreement with previous studies on allozyme analysis by Ivanova *et al.* (2010a, 2010b, 2012). They also confirm the

results of mitochondrial (Martimianakis *et al*. 2011) and microsatellite (Francis *et al*. 2014) DNA analyses concerning the differences in genetic characteristics of *A. m. macedonica* from Bulgaria compared to other *A. m. macedonica* and *A. m. carnica* populations on the territory of the Balkan Peninsula.

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