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Genetic analysis of *Apis mellifera macedonica* (type *rodopica*) populations selectively reared for purposive production of honey bee queens in Bulgaria

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Article info:
Received: 25 March 2016
In revised form: 10 April 2016
Accepted: 26 April 2016

ABSTRACT

The genetic polymorphism in selectively reared in Bulgaria, local honey bee populations of *Apis mellifera macedonica* subspecies (type *rodopica*), has been studied, using analysis of six enzyme systems (MDH-1, ME, EST-3, ALP, PGM and HK) corresponding to 6 loci. Totally 458 worker bees from 12 bee breeding bases for artificially inseminated queens were used for this study. All these stations are part of the National Bee Breeding Association which officially implements a National Program for sustainable beekeeping in Bulgaria. All of the six loci were found to be polymorphic. Only EST-3 locus was established as fixed in one of the investigated populations. Polymorphism with three alleles was ascertained for MDH, ME, ALP, PGM and HK loci and with five alleles for EST-3 locus. The most common alleles in almost all of the populations were MDH-1 100, ME 100, EST-3 100, PGM 100 and HK 100. Two private alleles (frequency < 0.05) were found for two of the populations. The calculated level of polymorphism was 88.33% in only one of the populations and 100% - in all others. The observed and expected heterozygosities (Ho and He) ranged from 0.157 to 0.250 and from 0.206 to 0.272, respectively. The estimated mean FST value from allozyme data was 0.035. On the bases of the allele frequencies of the studied allozyme loci the Nei's (1972) genetic distance was estimated. It ranged between 0.002 and 0.060 among the populations studied.

Key words: *Apis mellifera*, allozymes, genetic variability, queen bees' breeding

Introduction

The selection of honeybees in Bulgaria has a comparatively long history and traditions. Detailed researches into the morphological features of the local honey bees were carried out and the obtained results were used as a basis for organization of the selective work with bees in Bulgaria till 1990 (Lazarov, 1935, 1936; Velichkov, 1970; Petrov, 2010).

A novel stage in the selective work and the queen rearing process in Bulgaria started in 1999 when a new National Program for bee breeding has been announced. Its main purpose was to conserve the gene pool of the local Bulgarian honey bees which has proven biological and productive advantages and good adaptation to the specific local conditions (Petrov, 2010).

In order to characterize the race structure of the local honey bee, the populations from different plain and mountain regions of Bulgaria have been studied by usage mainly of morpho-ethological approaches (Petrov, 1993, 1995, 1997). In addition, some biochemical and molecular genetic researches of the polymorphism in some protein, isoenzyme and DNA systems have been performed since 1996 (Ivanova et al., 2007; Francis et al., 2014; Uzunov et al., 2014). The basis for selective work with bees in the country is the local Bulgarian honeybee named by Petrov (1995) as *Apis mellifera rodopica*.

DOI: 10.69085/jbb20161079

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According to the rules of the program, the import of foreign bee races was forbidden and the main used for bee selection method was the thoroughbred breeding in the frames of local Bulgarian honeybees. The queen bees have been reared either through natural mating or through instrumental insemination. The control for the biological and productive qualities is done by the National Bee Breeding Association and includes determination and estimation of the queen's fertility. It is fulfilled by the characterization of the brood quantity and quality, winter resistance, swarming inclination, aggressiveness level, honey and wax productivity, hygienic behaviour. The beekeepers in the country could purchase queens from the reproductive bases of the National Bee Breeding Association, all of which receive initial material for breeding as artificially inseminated thoroughbred queens.

The purpose of the present study was to investigate and analyze comparatively population-genetic characteristics of different *Apis mellifera macedonica* (type *rodopica*) populations selectively reared for purposive production of honeybee queens in Bulgaria. Data received in the investigation could be useful for needs of the honey bee selection in Bulgaria and for the conservation of local honey bees.

Materials and Methods

Honey bee samples

Samples were collected from reproductive bases in Bulgaria which belong to National Bee Breeding Association. Honey bee samples were from managed colonies of *Apis mellifera macedonica* subspecies (type *rodopica*) reared in Bulgaria. Honey bee individuals used for this study were from colonies with artificially inseminated queens. Totally 458 worker bees from twelve different local populations were tested in this investigation (Figure 1, Table 1). Ten colonies per a population, 3 to 6 individuals per a colony were included in the study. Collected worker bees were transported to the laboratory alive and stored at -20 ^{0}C until being used. The thorax homogenization and electrophoresis in polyacrylamide gel were performed according to Meixner et al. (2013).

Allozyme analysis

Six enzyme 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, electrophoretic conditions and histochemical staining used for each enzyme system were as it was described by Meixner et al. (2013).

Figure 1. *Sampling areas for the honey bee populations studied.*

Statistical Analyses

Allele frequencies, mean number of alleles per locus, proportion of polymorphic loci at the 95% level, observed (Ho) and expected (He) heterozygosity, deviation from the Hardy-Weinberg equilibrium and Nei's genetic distance (Nei, 1972), were calculated using GENALEX software package (Peakall & Smouse, 2006).

Results

The 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 three or five different alleles in the studied populations. Three alleles were detected at MDH-1 (MDH65, MDH80 and MDH100), ME (ME90, ME100 and ME106), ALP (ALP80, ALP90 and ALP100), PGM (PGM80, PGM100 and PGM114) and HK (HK87, HK100 and HK110) loci. Five alleles were detected at EST-3 (EST80, EST88, EST94, EST100 and EST118) locus (Figure 2 – a, b, c, d, e, f).

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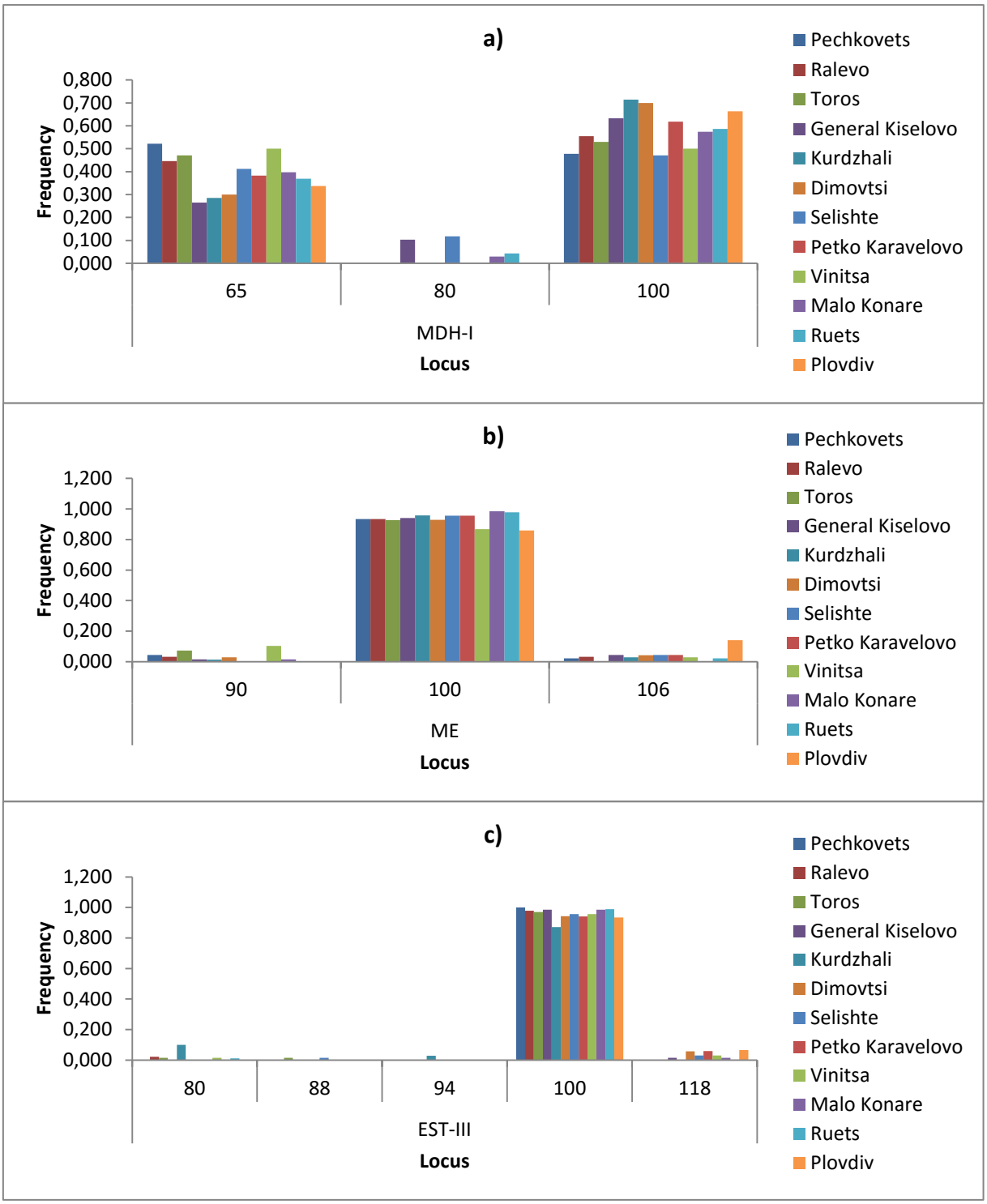

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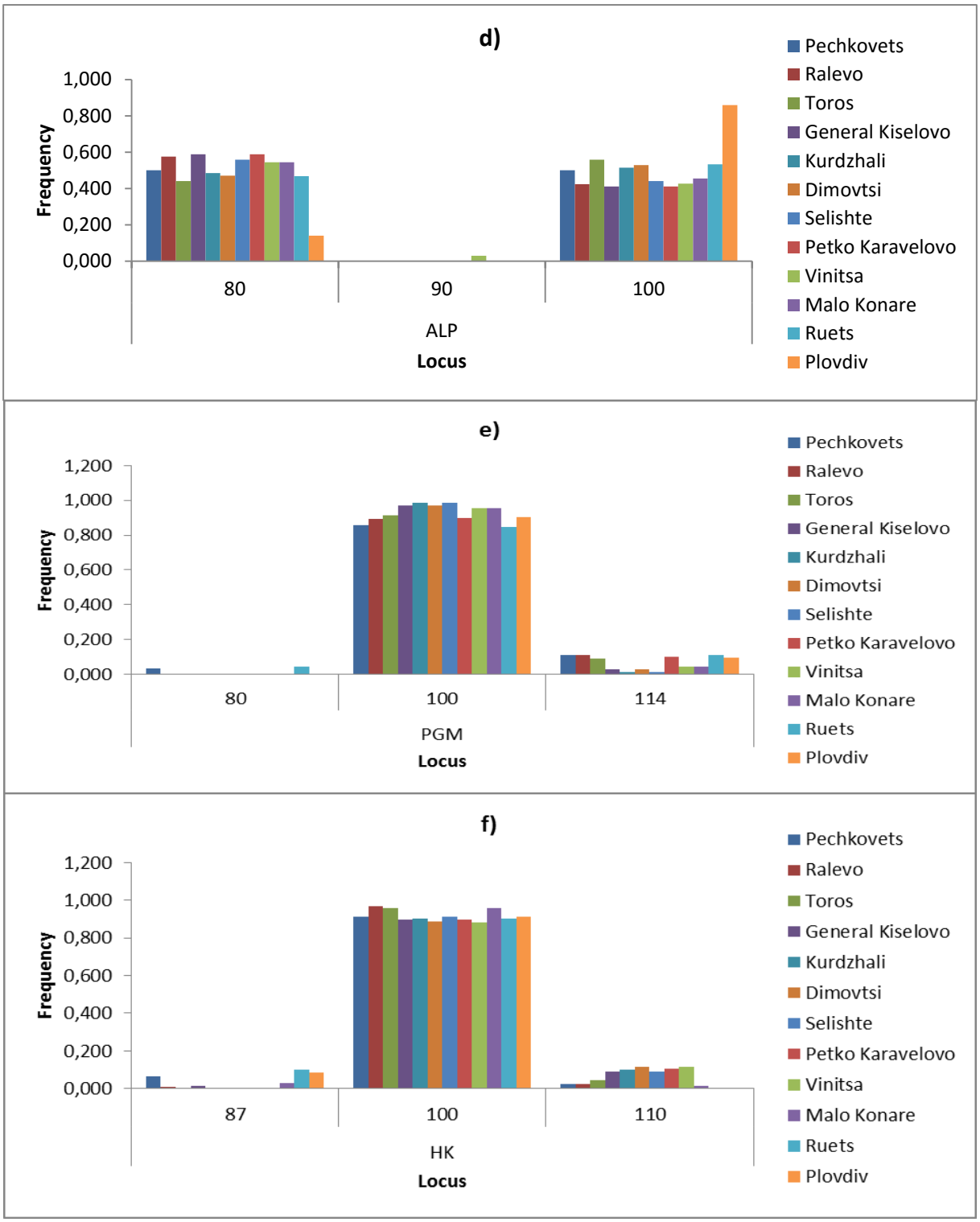

Figure 2. *Allele distributions for the loci studied with information about allele frequencies: Allele distribution and frequencies at MDH-I locus (a), Allele distribution and frequencies at ME locus (b), Allele distribution and frequencies at EST-III locus (c), Allele distribution and frequencies at ALP locus (d), Allele distribution and frequencies at PGM locus (e) and Allele distribution and frequencies at HK locus (f).*

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The mean number of alleles per locus was calculated to varied between 2.0 (Petko Karavelovo and Plovdiv) and 2.5 (General Kiselovo and Vinitsa). The effective number of alleles varied between 1.318 (Plovdiv) and 1.475 (Selishte) (Table 1).

Table 1. *Number of samples per population (N), number of alleles (Na), number of effective alleles (Ne), observed (Ho) and expected (He) heterozygosity (Standard errors are included).*

Population		N	Na	Ne	Ho	He
Pechkovets	Mean	46	2.333	1.444	0.207	0.256
	SE		0.333	0.181	0.109	0.084
Ralevo	Mean	46	2.333	1.404	0.221	0.234
	SE		0.211	0.180	0.110	0.084
Toros	Mean	34	2.167	1.411	0.196	0.238
	SE		0.167	0.182	0.082	0.083
General Kiselovo	Mean	34	2.500	1.411	0.196	0.232
	SE		0.224	0.192	0.090	0.088
Kurdzhali	Mean	35	2.333	1.388	0.229	0.238
	SE		0.211	0.155	0.080	0.075
Dimovtsi	Mean	35	2.167	1.385	0.157	0.237
	SE		0.167	0.156	0.086	0.074
Selishte	Mean	34	2.333	1.475	0.206	0.241
	SE		0.211	0.246	0.090	0.098
Petko Karavelovo	Mean	34	2.000	1.417	0.250	0.254
	SE		0.000	0.160	0.109	0.073
Vinitsa	Mean	34	2.500	1.474	0.245	0.272
	SE		0.224	0.184	0.077	0.080
Malo Konare	Mean	34	2.333	1.380	0.206	0.206
	SE		0.211	0.202	0.094	0.095
Ruets	Mean	46	2.333	1.451	0.236	0.254
	SE		0.211	0.190	0.110	0.088
Plovdiv	Mean	46	2.000	1.332	0.181	0.232
	SE		0.000	0.100	0.058	0.047
Total	**Mean**	**38.17**	**2.278**	**1.414**	**0.211**	**0.241**

The estimated percentage of polymorphic loci, using the 0.95 criterion, was 100% in almost all of the studied populations with exceptions of the Pechkovets population where the level of polymorphism was calculated as 83.3%.

In the present study, the observed and expected heterozygosities (Ho and He) ranged from 0.157 (Dimovtsi) to 0.250 (Petko Karavelovo) and from 0.206 (Malo Konare) to 0.272 (Vinitsa), respectively. The calculated mean observed and expected heterozygosities were 0.178 and 0.223, respectively (Table 1).

Two private alleles were observed in two of the studied populations – Kurdzhali (EST-380) and Vinitsa (ALP90).

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

The calculated F statistic which characterize additionally the heterozygosity in all populations is shown in Table 2. The mean heterozygosity in total populations for different loci (FIT) averaged to 0.250 (-0.016 – 0.503). Mean heterozygosity within subpopulation FIS was 0.221 (-0.087 – 0.490). The fixation coefficients of subpopulations for the loci studied within the total populations, measured as FST value, varied from 0.026 (ALP) to 0.065 (HK), with a mean of 0.035 (Table 2).

Table 2. *F-Statistics and estimates of Nm over all populations for each locus.*

Locus	F_{IS}	F_{IT}	F_{ST}	Nm
MDH-I	0.088	0.114	0.029	8.513
ME	0.424	0.440	0.029	8.467
EST-III	0.060	0.090	0.032	7.575
ALP	-0.090	-0.030	0.055	4.330
PGM	0.351	0.370	0.029	8.514
HK	0.520	0.532	0.025	9.726
Mean	0.226	0.253	0.033	7.854
SE	0.098	0.092	0.004	0.758

The mean value of gene flow (Nm) was calculated as 7.691 (3.577 – 9.5).

The genetic distances (Nei, 1972) ranged from 0.002 (between Ralevo and Malo Konare) to 0.060 (between Selishte and Plovdiv, Vinitsa and Plovdiv) and was in correlation with population assignment, summary of which is presented in Figure 3.

DOI: 10.69085/jbb20161079

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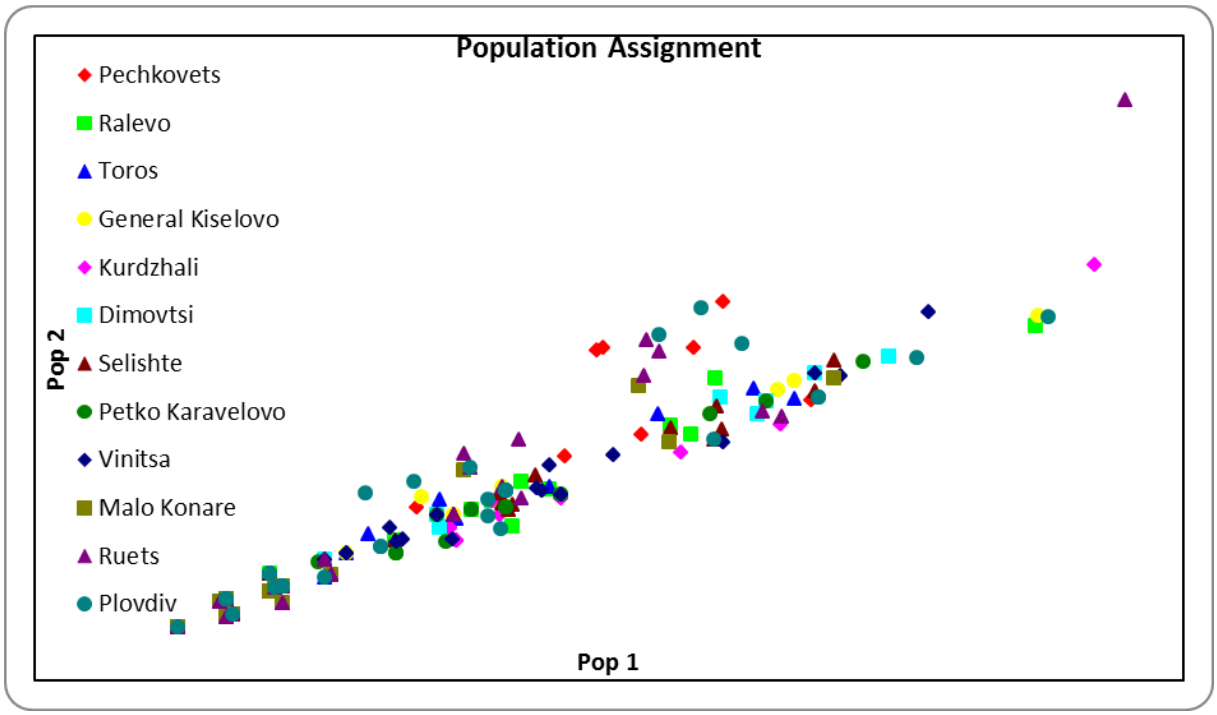

Figure 3. *Population assignment between individuals from the populations studied*.

Discussion

One of suitable parameter for investigating the genetic variability is gene heterozygosity. According to Ott's (2001), a polymorphic locus must have a heterozygosity of at least 0.10. In this aspect and on the base of calculated level of polymorphism in our investigation, it was found that all of the studied six alloenzyme loci had relatively high polymorphism (83.3% - 100%) with level of expected heterozygosity between 0.206 and 0.272 (Table 1).

There were found differences between total mean number of alleles per locus in the studied populations (2.000 – 2.500) and the effective allele number (1.332 – 1.475). Alleles with low frequencies contribute slightly to the effective number of alleles, so a presence of discrepancy between "number of alleles per locus" and "effective number of alleles per locus" gives information about the presence of alleles with lower (not equal) frequency in the population.

Evaluation of Fst values for almost all of the loci studied in our investigation (MDH-1, ME, EST-3, PGM and HK) demonstrated low levels of genetic differentiation (0.025 – 0.032) in accordance with Hartl & Clark (2007) data. Only for ALP locus this value was calculated as 0.055, which demonstrated a moderate level of genetic differentiation (Table 2). The estimated mean FST value was 0.033 which shows that 3.3% of the overall observed genetic diversity was among populations and respectively 96.7% within populations.

The data received in our investigation showed that the gene flow (Nm) was greater than 2 (mean – 7.854) for all of the studied loci (4.330for the ALP locus – 9.726 for the HK locus), which indicated very low genetic differentiations among the studied populations (Table 2).

In accordance with this, the assignment test showed a high level of consolidation for the all studied populations (Figure 3).

Private alleles found could be used as genetic markers for distinguishing of some honey bee populations.

The results of this research give new detailed population genetic information concerning selectively controlled *Apis mellifera macedonica* (type *rodopica*) populations which have been successfully used for purposive production of honeybee queens in Bulgaria. These results would be useful for more precise characterization of the local honey bee by giving the information about suitable genetic markers for future selection.

DOI: 10.69085/jbb20161079

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Acknowledgement

This study was funded by the Plovdiv University Research Fund by the contract NI 15 FS 017.

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DOI: 10.69085/jbb20161079