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# Alloenzyme genetic characterization of *Apis mellifera* (*Hymenoptera: Apidae*) colonies from Bulgaria with different hygienic behaviour

# Aloenzimna genetichna harakteristika na pchelni semeystva *Apis mellifera* (*Hymenoptera: Apidae*) ot Bulgaria s razlichno higienno povedenie

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## ABSTRACT

The genetic variability in 25 honey bee colonies from different regions of Bulgaria with different hygienic behaviour (highly hygienic, hygienic and non-hygienic) has been studied. Alloenzyme analysis of two systems (MDH-1 and Est-3) corresponding to 2 loci was used in order to characterize the colony polymorphism. Totally 1,150 worker bees were included in this investigation. MDH-1 locus was found to be polymorphic in all of the studied colonies, having two alleles – MDH-$1^{65}$ and MDH-$1^{100}$. The Est-3 locus was fixed in ten of the investigated colonies. Polymorphism with total presence of four alleles of this locus (Est-$3^{80}$, Est-$3^{88}$, Est-$3^{100}$ and Est-$3^{118}$) was found in the other studied colonies. The calculated polymorphism was 50% in the non-hygienic and 100% in the highly hygienic and hygienic colonies. The observed and expected heterozygosities ($H_o$ and $H_e$) ranged from 0.296 to 0.354 and from 0.28 to 0.332 in non-hygienic and highly hygienic groups, respectively. The calculated mean observed and expected heterozygosities were 0.32 and 0.307, respectively. The calculated $F_{st}$ and $N_m$ levels demonstrated lower differentiation between highly hygienic and hygienic colonies and higher differentiation between highly hygienic and non-hygienic colonies. Dissimilarities between levels of polymorphism, heterozygosity, $F_{st}$, $N_m$ and allele frequencies in the studied groups of colonies with different hygienic behaviour were found and discussed. The results of the present study provide new information concerning relations between hygienic behaviour and alloenzyme characteristics which could be used for future selection with honey bees in Bulgaria.

**Keywords:** alloenzyme, *Apis mellifera*, genetic polymorphism, hygienic behaviour

## ABSTRAKT

Obekt na nastoyashtoto izsledvane e genetichnata izmenchivost, ustanovena pri rabota s 25 pchelni semeystva ot razlichni rayoni na Bulgaria s razlichno higienno povedenie (Visoko higienichni, higienichni i ne-higiennichni). Izyaveniyat polimorfizam e harakteriziran na bazata na aloenzimen analiz po dve sistemi (MDH-1 i Est-3), saotvetstvashti na dva polimorfni lokusa. Obshto 1150 pcheli rabotnichki sa vklyucheni v izsledvaneto. Ustanoveno e, che MDH-1 lokusat e polimorfen vav vsichki izsledvani pchelni semeystva i e predstaven ot dva alela - MDH-$1^{65}$ i MDH-$1^{100}$. Est-3 lokusat e fiksiran v deset ot izsledvanite kolonii. Po tozi lokus e konstatiran polimorfizam s prisastvie obshto na chetiri alelni varianti (Est-$3^{80}$, Est-$3^{88}$, Est-$3^{100}$ i Est-$3^{118}$) za genofonda na ostanalite pchelni semeystva. Izchisleniyat polimorfizam e mezhdu 50% i 100%. Ustanovenata i ochakvanata heterozigotnost ($H_o$ i $H_e$) varira saotvetno ot 0.296 do 0.354 i 0.28

ot 0.332 pri ne-higienichnite i visoko higienichnite grupi. Izchislenite sredna nablyudavana i ochakvana heterozigotnosti sa saotvetno 0.32 i 0.307. Izchislenite niva na $F_{st}$ i $N_m$ pokazvat po-niska diferentsiatsia mezhdu visoko higienichnite i higienichnite semeystva i visoka diferentsiatsia mezhdu visoko higienichnite i ne-higienichnite semeystva. Konstatirani i obsadeni sa razlichia mezhdu nivata na polimorfizam, heterozigotnost, $F_{st}$, $N_m$ i alelnite chestoti v izsledvanite grupi pchelni semeystva s razlichno higienno povedenie. Rezultatite ot nastoyashtoto prouchvane davat nova informatsia otnosno zavisimostta mezhdu nivata na higienno povedenie i aloenzimnite harakteristiki, koito biha mogli da se izpolzvat v badeshti deynosti po selektsia na medonosnite pcheli v Bulgaria.

**Klyuchovi dumi:** aloenzimi, medonosna pchela, genetichen polimorfizam, higienno povedenie

## INTRODUCTION

Hygienic behaviour in honeybees is considered one of the main factors of genetic resistance of the bee organism. The cleaning instinct is inherited in the offspring and affects directly the health status of bee colonies. It is expressed in detecting and discarding infected and dead larvae and bees outside the hive, thereby limiting the spread of infection within the bee colony.

Knowledge on hygienic behaviour and its relationship with a number of biological factors is crucial for the selection and creation of bee breeds resistant to a number of diseases (Milne, 1982; Taber, 1986; Taber and Gilliam, 1987, 1988; Fukae et al., 1990; Choi et al., 1991; Spivak and Gilliam, 1993; Southwick, 1994a, b; Hornitzky, 1995; Petrov, 1997; Jeliazkova and Gurgulova, 2003, Darkazanli, 2008). After a Regulation prohibiting the use of sulfonamides and antibiotics in beekeeping (Council Regulation 2377/90, 1990) came into effect in the European Union, respectively Bulgaria, studies are aimed at breeding colonies with high level of natural immunity have become especially topical.

Generally, both genotype and environment are determinative for hygienic behaviour of honey bees. Local adaptation influences also its expression. In order to study this relation different approaches could be used. In the present investigation was to investigate comparatively population-genetic characteristics in honey bee colonies with highly hygienic, hygienic and non-hygienic behaviour on the base of alloenzyme analysis on two systems – malate dehydrogenase and esterase. Such kind of study could give more detail information concerning the relation between the hygienic behaviour and alloenzyme polymorphism in different honey bee colonies and also would be used for future selection with honey bees in Bulgaria.

## MATERIAL AND METHODS

### *Testing colonies for level of manifestation of hygienic behaviour*

The study was conducted in the period 2015 and 2016 during the active bee season. The testing for level of expression of the hygienic behaviour included 25 bee colonies from 10 apiaries in different regions of the country. Bee colonies equal in power of the local breed of *Apis Mellifera* L. have been used.

When testing bee colonies for hygienic behaviour a method modified by Gurgulova et al. (2003) has been applied – different from the method of Taber and Gilliam (1988) and similar to that of Petrov (1997). Depending on the time and the extent of cleaning the marked section 5 x 5 cm (100 worker bee cells) colonies are divided into three groups: highly hygienic – colonies which on the 24$^{th}$ hour after puncturing clean over 95% of the cells in the marked section; hygienic - colonies which on the 48$^{th}$ hour after puncturing clean over 95% of the marked section; non-hygienic - those which clean less than 95% of the cells in the section by the 48$^{th}$ hour.

### *Alloenzyme analysis*

Totally about 1,150 honey bee samples, collected from the mentioned managed colonies were tested in three groups concerning their hygienic behaviour – highly hygienic (HH), hygienic (H) and non-hygienic (NH). Number of collected worker bees per a colony was between 40 and 50. After collecting worker individuals

were stored at -20 °C until used for electrophoresis. Total homogenization and electrophoresis in polyacrylamide gel were done according to Meixner et al. (2013).

Two enzymic systems were studied: MDH (malate dehydrogenase, EC 1.1.1.37) and EST (esterase, EC 3.1.1). Buffers and electrophoretic conditions for each enzyme system used in the study were as in Shaw and Prasad (1970). Enzyme activities were visualized by histochemical staining according to Harris and Hopkinson (1976). Allele products were designed with respect to their relative mobility, as the mobility of the most common alloenzyme was used as standard (mobility 100).

***Statistical analysis***

The results of alloenzyme analysis were statistically performed using GenAlEx 6.5 (Peakall and Smouse, 2012). On this base frequency-based statistics and population assignment were calculated. Mean number of alleles per locus, proportion of polymorphic loci, observed ($H_o$) and expected ($H_e$) heterozygosity were comparatively analyzed for highly hygienic, hygienic and non-hygienic groups of honey bee colonies.

## RESULTS

The enzyme systems studied correspond to two loci – MDH-1 and Est-3. Two alleles were found at MDH-1 and four alleles – at Est-3 loci (Table 1).

**Table 1.** Allele frequencies and sample size per loci and groups studied

| Locus | Allele/n | Highly hygienic | Hygienic | Non-hygienic |
|---|---|---|---|---|
| MDH-1 | N | 414 | 322 | 414 |
| | 65 | 0.402 | 0.371 | 0.471 |
| | 100 | 0.598 | 0.629 | 0.529 |
| Est-3 | N | 414 | 322 | 414 |
| | 80 | 0.071 | 0.016 | 0.004 |
| | 88 | 0.017 | 0.008 | 0 |
| | 100 | 0.901 | 0.919 | 0.969 |
| | 118 | 0.011 | 0.057 | 0.028 |

Data concerning allele frequencies, number of alleles per locus, effective alleles, observed ($H_o$) and expected ($H_e$) heterozygosity is presented in Tables 1, 2 and 3.

The results of the current investigation showed that more frequent alleles for all studied groups of honey bee colonies were MDH-$1^{100}$ (0.529 – 0.629) and Est-$3^{100}$ (0.901 – 0.969). It is important to note that in the gene pool of the group with non-hygienic colonies the alleles MDH-$1^{65}$ and Est-$3^{100}$ were with the highest frequency (0.471 and 0.969, respectively) in comparison with the other two groups – Fig. 1 and 2. Meanwhile, the allelic diversity in the gene pool of the highly hygienic and hygienic groups was greater, including four alleles – Est-$3^{80}$, Est-$3^{88}$, Est-$3^{100}$ and Est-$3^{118}$. The comparison of the rare alleles' frequencies in these two groups showed that Est-$3^{80}$ was with highest frequency (0.071) in the gene pool of the highly hygienic group and Est-$3^{118}$ – with highest frequency (0.057) in the gene pool of the hygienic group – Table 1, Figures 1 and 2.

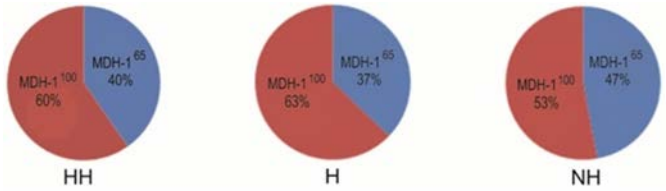


**Figure 1.** Allele Frequency at MDH-1 locus for highly hygienic (HH), hygienic (H) and non-hygienic (NH) colonies

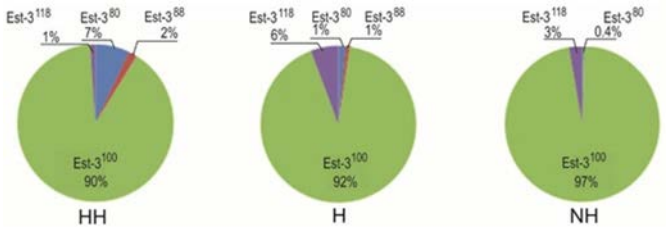


**Figure 2.** Allele Frequency at Est-3 locus for highly hygienic (HH), hygienic (H) and non-hygienic (NH) colonies

The calculated mean number of alleles per locus was 2.5 for non-hygienic group and 3 – for both groups of hygienic colonies. The number of effective alleles varied from 1.527 (for hygienic) to 1.575 (for super hygienic) (Tables 2 and 3).

**Table 2.** Number of individuals (N), number of alleles ($N_a$), number of effective alleles ($N_e$), observed ($H_o$) and expected ($H_e$) heterozygosity and fixation index (F)

| Pop | Locus | N | $N_a$ | $N_e$ | $H_o$ | $H_e$ | F |
|---|---|---|---|---|---|---|---|
| Super hygienic | MDH-1 | 414 | 2 | 1.926 | 0.51 | 0.481 | -0.06 |
| | Est-3 | 414 | 4 | 1.224 | 0.198 | 0.183 | -0.084 |
| Hygienic | MDH-1 | 322 | 2 | 1.875 | 0.469 | 0.467 | -0.005 |
| | Est-3 | 322 | 4 | 1.178 | 0.149 | 0.151 | 0.015 |
| Non-hygienic | MDH-1 | 414 | 2 | 1.993 | 0.541 | 0.498 | -0.086 |
| | Est-3 | 414 | 3 | 1.065 | 0.051 | 0.061 | 0.169 |

**Table 3.** Mean and standard error (SE) over loci for each population and data concerning percentage of polymorphic loci (P)

| Pop | | N | Na | $N_e$ | $H_o$ | $H_e$ | P (%) | F |
|---|---|---|---|---|---|---|---|---|
| Highly hygienic | Mean | 414 | 3 | 1.575 | 0.354 | 0.332 | 100 | -0.072 |
| | SE | 0 | 1 | 0.351 | 0.156 | 0.149 | | 0.012 |
| Hygienic | Mean | 322 | 3 | 1.527 | 0.309 | 0.309 | 100 | 0.005 |
| | SE | 0 | 1 | 0.349 | 0.16 | 0.158 | | 0.01 |
| Non-hygienic | Mean | 414 | 2.5 | 1.529 | 0.296 | 0.28 | 50 | 0.042 |
| | SE | 0 | 0.5 | 0.464 | 0.245 | 0.219 | | 0.127 |
| Grand mean and standard error over loci and populations | | | | | | | | |
| Total | Mean | 383.333 | 2.833 | 1.544 | 0.32 | 0.307 | | -0.008 |
| | SE | 19.395 | 0.401 | 0.175 | 0.086 | 0.08 | | 0.039 |

Difference between mean number of alleles per locus – 2.833 and mean number of effective alleles ($N_e$=1/(1-$H_{exp}$)) – 1.544, in the studied groups was found. It should be mentioned that the number of different alleles and the number of these of them which frequency was higher than 5% was larger for highly hygienic and hygienic colonies in comparison with non-hygienic (Figure 3).

The estimated percentage of polymorphic loci (P=0.95) was 50% in non-hygienic and 100% in others two groups.

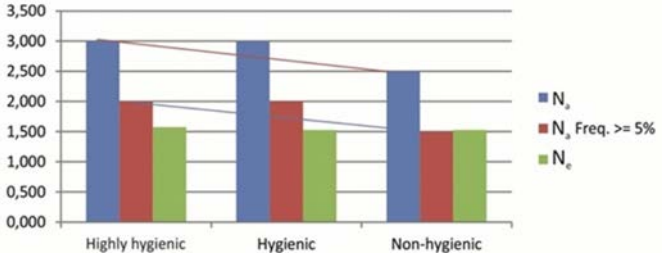


**Figure 3.** Number of different alleles, number of different alleles with frequency ≥5% and number of effective alleles ($N_e$=1/(1-$H_{exp}$) per locus

In the present study, the observed and expected heterozygosities ($H_o$ and $H_e$) ranged from 0.296 to 0.354 and from 0.28 to 0.332 in non-hygienic and highly hygienic groups, respectively (Table 3). The calculated mean observed and expected heterozygosities were 0.32 and 0.307, respectively.

It was interesting that the levels of observed and expected heterozygosities for Est-3 locus (0.051 – 0.198 and 0.061 – 0.183, respectively) were much lower than for MDH-1 locus (0.469 – 0.51 and 0.467 – 0.498, respectively). The results of the present study showed the lowest values of $H_o$ and $H_e$ of non-hygienic colonies and highest for highly hygienic colonies (Table 2).

The calculated F statistic gives additional information about the levels of heterozygosity in the investigated groups (Tables 3 and 4). In the present investigation $F_{st}$ values for both studied loci (0.005 – 0.012) demonstrated low levels of genetic differentiation. The fixation index F varied from 0.042 to -0.072, with a mean of -0.008 and demonstrated slight excess of heterozygotes and low level of genetic differentiation between the studied groups (Table 3).

Data received in the present investigation showed levels of $N_m$ (gene flow) greater than 2 for both studied loci, which indicated low genetic differentiations among the studied honey bee groups. The Nm value between highly hygienic and non-hygienic colonies was 20.275 and between highly hygienic and hygienic colonies – 52.578 (Table 4) which defined more considerable differentiation between first couple of groups (HH and NH), and lower differentiation between the second one (HH and H).

There were not found significant deviations of genotype frequencies from Hardy-Weinberg expectations at both loci (with exception of Est-3 for non-hygienic colonies) in the groups studied (P≥0.1). Chi-Square tests showed slight deviations generally in favor of the heterozygotes, which is in correlation with data concerning $H_o$ and $H_e$, and F statistics (Tables 3 and 4).

**Table 4.** Pairwise Population fixation index Values ($F_{st}$) and Estimates of gene flow ($N_m$)

| Group 1 | Group 2 | Fst | Nm |
|---|---|---|---|
| Highly hygienic | Hygienic | 0.005 | 52.578 |
| Highly hygienic | Non-hygienic | 0.012 | 20.275 |
| Hygienic | Non-hygienic | 0.009 | 26.855 |

## DISCUSSION

In the present study both of studied loci (MDH-1 and Est-3) were polymorphic in all of the studied groups of honey bee colonies with different hygienic behaviour. The results showed that the highest allele frequencies of MDH-$1^{100}$ (53-63%) and of Est-$3^{100}$ (90-97%) were not in relation with the levels of hygienic behaviour expression.

Some differences were found in the allele frequencies of Est-3 locus depending on the hygienic behaviour. In the non-hygienic colonies Est-$3^{88}$ allele was absent and Est-$3^{80}$ allele was with the lowest frequency (less than 1%) in comparison to the other two groups of colonies – highly hygienic and hygienic.

The mean number ($N_a$) of alleles per locus varied from 2.5 (non-hygienic group) to 3 (highly hygienic and hygienic colonies).

Results showed that the alleles with low frequencies contribute very little to the number of effective alleles which were 1.575 for highly hygienic colonies, 1.527 and 1.529, respectively for hygienic and non-hygienic colonies. Bee colonies with higher expression of hygienic behaviour (highly hygienic and hygienic) had a greater number of different alleles. Also, number of alleles with frequency higher than 5% in hygienic colonies was greater in comparison with non-hygienic.

A suitable parameter for investigating the genetic variability within and between the populations is the heterozygosity. According to the Ott' opinion (2001), a polymorphic locus must have a heterozygosity of at least 0.1. It was seen an important tendency in the studied groups – the levels of observed ($H_o$) and expected ($H_e$) heterozygosities were the lowest in non-hygienic colonies, intermediate – in hygienic colonies and the highest – in the highly hygienic colonies.

The calculated F statistic gives additional information about the levels of heterozygosity in the investigated three groups bee colonies – highly hygienic, hygienic and non-hygienic. According to Hartl and Clark (2007), $F_{st}$ levels between 0 and 0.05 indicate low genetic differentiation, between 0.05 and 0.15 – moderate, between 0.15 and 0.25 – high genetic differentiation and levels larger than 0.25 designate highly significant genetic differentiation. The results of this study (0.005 to 0.012) correspond to low genetic differentiation.

The parameter gene flow - $N_m$ gives information about genetic divergence or genetic similarity of subpopulations due to the gene flow between them. $N_m$ values lower than 2 demonstrate considerable genetic differentiation among subpopulations. In accordance with $N_m$ values, pairwise group $F_{st}$ values were as follows: 0.005 – between HH and H; 0.009 – between H and NH; 0.012 – between HH and NH, which confirmed the mentioned above tendency.

## CONCLUSIONS

Differences in allele frequencies for MDH-1 and Est-3 loci, levels of polymorphism and levels of heterozigosities were found for the compared honey bee colonies with varied hygienic behaviour. The results of this study give information concerning the possible relations between the hygienic behaviour and alloenzyme polymorphism in different honey bee colonies and could be used for future selection with honey bees in Bulgaria.

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