Thesis of the Ph.D. dissertation

STUDING THE FEATURES OF THE BORDERING SUBSPECIES IN THE HUNGARIAN HONEY BEE POPULATIONS (*APIS MELLIFERA CARNICA PANNONICA* POLL.) WITH GENETIC AND MORPHOLOGICAL METHODS

Author:
Erika Péntek-Zakar
Ph.D. candidate

Supervisor:
Dr. Szilvia Kusza Ph.D.

UNIVERSITY OF DEBRECEN
Doctoral School of Animal Husbandry

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I. BACKGROUND AND AIMS OF THE PH.D. THESIS

Apiary is a unique part of the animal husbandry. The lives of bees are more dependent on the natural environment and accommodate to the ecological farming than the other forms. Not only because of their food producing function, but also because of the indirect benefits of pollination.

The pollination activities of the nearly half million bee families are invaluable in Hungary as well. Besides maintaining biodiversity it helps the growth of the average yield of agricultural cultures (Szalainé, 2002). Hungarian apiary gives 1% of the gross agricultural production value, and nearly 3% of the animal husbandry (47/2010. (XII. 31.) VM).

As the bees copulate in natural way and outdoors, the male remains unknown and its identification is not possible. Therefore it is very difficult to control copulation, so the gene fluxation among subspecies is usual (Franck et al., 1998). One of the requirements of identifying both parents and with this introducing classic breeding methods is to do local research on the genetic variations of the bee population, recognizing potential gene contamination, and adapting and using the results in apiary, as well. It has been widespread since the beginning of the 20th century to trade with queens, among which mainly the quality species dominate, such as *Apis mellifera ligustica* in Italy and *Apis mellifera carnica* in the former Yugoslavia and in North West Europe (Peer, 1957).

Currently the only acknowledged and breedable bee in Hungary is the Pannon form of the Carniolan subspecies (*Apis mellifera carnica pannonica*), and its breeding is being monitored and controlled. Nevertheless, besides the apiary products' export we need to count on the export and import of the queens, and the potential natural hybridisation. As a result of this we need to count on the genome change of the *Apis mellifera carnica pannonica* that has greatly adapted to the ecological conditions of the Carpathian Basin, has got acclimatised during the centuries and has been formed by queen breeders (Szalainé, 2000).

To prove our hypothesis we have completed genetic and morphological studies. These two methods perfectly supplement each other. Because only phenotype analysis would not give information about genetic structure of each population, the degree of diversity from other genotypes. Most of the genetic substance is not shown in phenotypes, so one of the main aims of the gene sustaining breeding is to find, keep and protect the alleles. The molecular genetic markers show polymorphism to a great extent, and therefore are perfect tools for genetic analysis of individuals and stocks, as well as comparing genetic populations.

1.1. Aims of the research

Proving the presence of the unknown bee subspecies in the Hungarian honey bee populations using genetic markers (mitochondrial DNA and microsatellite) and morphological parameters.

- Haplotype investigation of the domestic honey bee populations, foreign control stocks and sequences from gene bank with the help of one section of the mitochondrial cytochrome-oxidase I region.

- Genetic diversity research of the domestic Carniolan subspecies and its comparison with control populations using microsatellite markers.

- The description of the native honey bee populations using the generally accepted three morphological parameters (tergite colour, proboscis length and cubital index) and K19 angle which is a new parameter in the Hungarian morphological examination practice.

II. METHODS

In Hungary we took samples from 16 apiaries in the summer of 2010. From every apiaries, 5 families; ten from every families, 5-7 days old worker larvae were collected prior to covering. We also analysed the following full-grown subspecies: *A. m. ligustica* (n=15) from Italy (Bologna), *A. m. adansonii* (n=15) from Liberia (Jibloo), *A. m. iberica* (n=10) from Spain (Galicia, Cantabria, Navarra, Murcia, Castilla Leon), *A. m. mellifera/carnica* (n=50) from Poland (Krakkow, Bialowieza, Siedlice, Bydgoszcz, Wroclaw), and individuals of the Buckfast hybrids (n=20) were also available from our country (Figure 1).

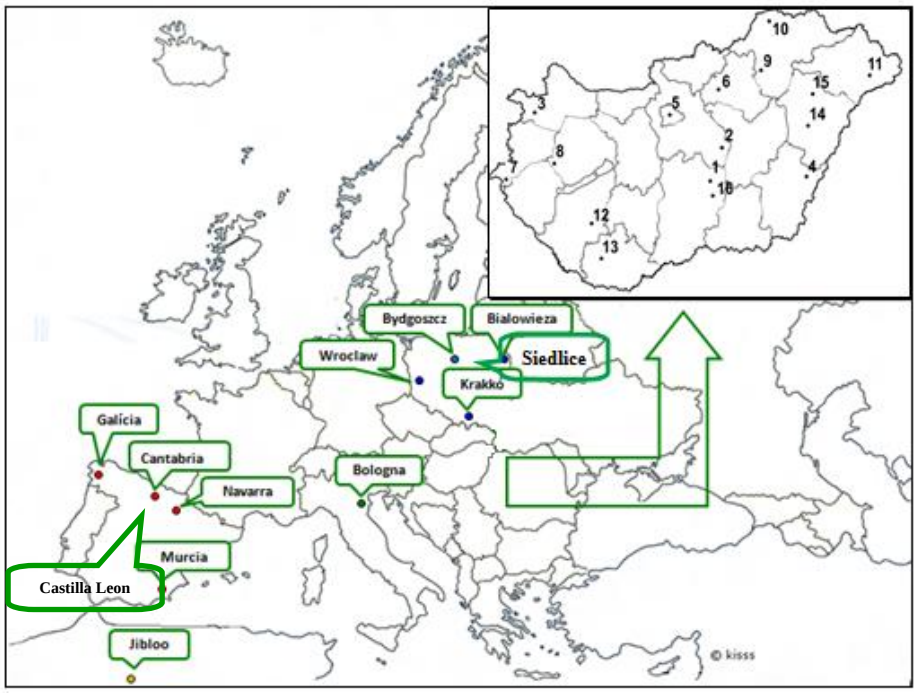


Figure 1: The apiaries studied in abroad and Hungary

The worker larvae were carried separately to the laboratory in 1,5 µl eppendorf tubes, and then were kept at -20 °C until the genomic DNA extract.

2.1. Mitochondrial DNA study

For the mitochondrial DNA study we used two worker larvae from each family, from each apiary and from three families (n=96), from the foreign populations we used an average of ten individuals (n=84). Furthermore, 53 reference sequences were available from the NCBI gene bank.

We amplified a defined section of the mitochondrial DNA's COI gene with PCR (Polimerase Chain Reaction) with the use of the following primers designed by us: forward (5'-CTGATATAGCATTCCCCCGAATA-3') and reverse (5'-AGAATTGGATCTCCACGTCCTA-3').

The cleaned PCR products in every case were kept at -20 °C until shipping. The sequencing was led by the Eurofins MWG Operon (Germany) company.

We used the following programs for the statistical analysis of the data: BioEdit, ClustalX, DnaSP v. 5.10., Mega v. 5, Network v. 4.6.1.1 and GenAlex v. 6.4. For tracking the different positions we used the full *Apis mellifera ligustica* (Acc. no.: L06178.1) mitochondrial genome from the NCBI gene bank. With the help of the FindModel programme that is available on the internet, we established the ideal Hasegawa-Kishino-Yano (HKY) plus Gamma model for the sequences. Knowing the suitable algorithm, we designed a Maximum-Likelihood phylogenetic tree. While doing the phylogenetic tree, as a control we chose a wild bee species (*Trigona amalthea*, Acc. no.: AF214669) that is genetically different from the domestic bees.

2.2. Microsatellite study

For the microsatellite study we worked on three bee larvae per families from the Hungarian apiaries, from each population, from five families (n=236) (Buckfast line n=10), and 106 foreign samples.

For the PCR reaction we used the nine most widely used microsatellite markers – according to the available research information (A7, A113, A107, A28, A88, A14, A35, A(B)24) (Estoup et al., 1995), A43 (Garnery et al., 1998).

The fragment analysis was carried out by the laboratory of Biomi Kft (Gödöllő). The nine microsatellite markers were run in three multiplexes. The allele results were read via PeakScanner v. 1.0 program (Applied Biosystem, USA). To analyse the evaluation of the statistical data we used Arlequin v. 3.1, GenAlex v. 6.4, Fstat v. 2.9.3, PopGene v. 1. 32. and Structure softwares. From the output files of the PopGene programme we generated an output file using Phylip program packet (Neighbour programme). We visualized the received UPGMA phylogenetic tree with the TreeView program. With the Barrier program we could demonstrate the genetic barriers among the examined populations – this was done by using the Delaunay triangle method and Monmonier algorithm based on pairwise F_{ST} values.

2.3. Morphological study

For the Hungarian morphological study we used the same samples collected from the 16 apiaries, from each apiary, from five families, 50-50 full-grown drones from each family.

In addition to the general morphological study parameters in Hungary (tergite colour, proboscis length and cubital index) we applied the K19 angle that is suitable here according to the professional literature.

The characteristic of the Carniolan bee is a grey or dark abdomen. In this study we assessed the presence or absence of other possible abdomen colour variations (yellow). According to the (Bee Efficiency Study Kodex, 2003) the following categories can be distinguished:

- S – the colour of the abdomen ring's exoskeleton is dark
- I – on the first tergite there are yellow stains on the two sides
- II – on the first tergite there is a yellow stripe (max.: 4%)
- III – on the second tergite the yellow stripe is also present (exclusive reason)
- IV – there is a yellow stripe on the third tergite as well (exclusive reason)

We cut of the head 20 individuals of a family during the proboscis' autopsy. We aimed to avoid breaking the proboscis during the autopsy, and also that the end of it would come off. The anatomized proboscis was held in alcohol (70%) until performing the examination to make sure they do not dry out. We stabilized the proboscis with sellotape to the plates. We measured their length with microfilm readers and rulers. The size of the proboscis (submentum+mentum+glossa) can be 6,50-6,80 mm according to the acknowledged species standard, but it has to be minimum 6,50 mm.

For examining the cubital index (CI) on the first wing, we again dissected the first wings of 20 individuals from the families, and then kept these in alcohol (70%) until the examinations. According to the species standard the value of the cubital index is 2,30-3,00. We stuck the wings on a 12,2 x 3,9 cm glass plate with white labels. On the white labels we had the number of the family and the breeder's code. We stuck eight individual's wings in one row, and six individuals' first wings in one column, so on one plate there were 48 wing dissections available. After the gluing and drying we used a Carl Zeiss microscope. The tools needed for the measures were provided by the following group: Institute for Small Animal Research and Co-ordination Centre for Gene Conservation, Department of Honey Bee Keeping.

For the recording of the K19 angle on the first wing we used Optika microscope and the related Optika Micro Image Analysis Software, which was offered to us by the University of Debrecen Department of Nature Conservation, Zoology and Game Management.

The values of each parameter were recorded and analysed in an Excel table.

III. MAIN OBSERVATIONS OF THE THESIS

3.1. Results of the mitochondrial DNA study

We found 17 haplotypes in the cytochrome-oxidase I (CO I) mitochondrial region's defined section in the Hungarian and foreign control individuals. We also demonstrated that the Ht 9 is dominant in Central Europe and its frequency is growing towards south. Ht 8, Ht 11 and Ht 12 appeared as a new haplotype in Hungary, it had not been available on the NCBI database. Ht 4, Ht 13 and Ht 14 were new in Spain, and Ht 5, Ht 6 and Ht 7 in Poland. In Liberia the population (n=10) is the same in all samples, the member of Ht 15; but the Buckfast line's each individual (n=10) faded into the most common haplotype of Central Europe that is Ht 9.

Table 1: mtDNA diversity index of the studied populations
n element number, *N hap* haplotype number, *Hd* haplotype diversity, *Pi* nucleotide diversity

Populations	Subspecies	n	*N hap*	*Hd*	*Pi*
Hungary	*A. m. carnica*	96	7	0.296±0.060	0.0009±0.001
Liberia	*A. m. adansonii*	10	1	0.000±0.000	0.000±0.000
Spain	*A. m. iberica*	10	4	0.533±0.180	0.007±0.004
Poland-Bydgoszcz	*A. m. mellifera*	10	5	0.756±0.130	0.007±0.004
Poland-Krakkow	*A. m. carnica*	10	3	0.600±0.131	0.001±0.001
Poland-Bialowieza	*A. m. carnica*	9	2	0.389±0.164	0.001±0.001
Poland-Wroclaw	*A. m. mellifera*	5	2	0.356±0.159	0.004±0.003
Poland-Siedlice	*A. m. carnica*	10	2	0.400±0.237	0.001±0.001
Hungary	Buckfast line	10	1	0.000±0.000	0.000±0.000
Italy	*A. m. ligustica*	10	2	0.356±0.159	0.004±0.003

In accordance with the element number the most haplotypes were detected in the Hungarian population (*N hap*=7), the greatest diversity was found in the Polish population of Bydgoszcz.

The haplotype diversity showed medium or above medium values, which is also true for the nucleotide diversity. The Liberian population was an exemption, as well as the individuals we examined from the Buckfast line (Table 1).

We determined the 15 variable positions of the 17 haplotypes we studied, and this was done for the reference sequences (Accession number: L06178.1 *Apis mellifera ligustica*

complete mitochondrial genome). In 11 cases the nucleotide exchanges were a transitions, in three cases they were transversions (C/A, T/A, A/T), in one case in position 2169 both transition (G/A) and transversion (G/C) took place.

We established that the most different from the Hungarian and European populations we examined based on the mitochondrial DNA is the Liberian population. Significant genetic distance is shown from the Hungarian bee population in the Bydgoszcz (0.870) and Wroclaw (0.883) populations, as well. This hypothetically happened because Poland is bordering the *A. m. mellifera* and *A. m. carnica* subspecies.

The least divergence was revealed among the following populations: Hungarian, Polish Bialowieza (0.061), Siedlice (0.042) and Krakkow (0.160). The low divergence could mean that just as the Hungarian, the three Polish populations are also Carniolan subspecies, and most of their individuals belong to the „C" origin line. The low distance measures between the Italian and Hungarian populations (0.277) also indicate the common origin (Table 2).

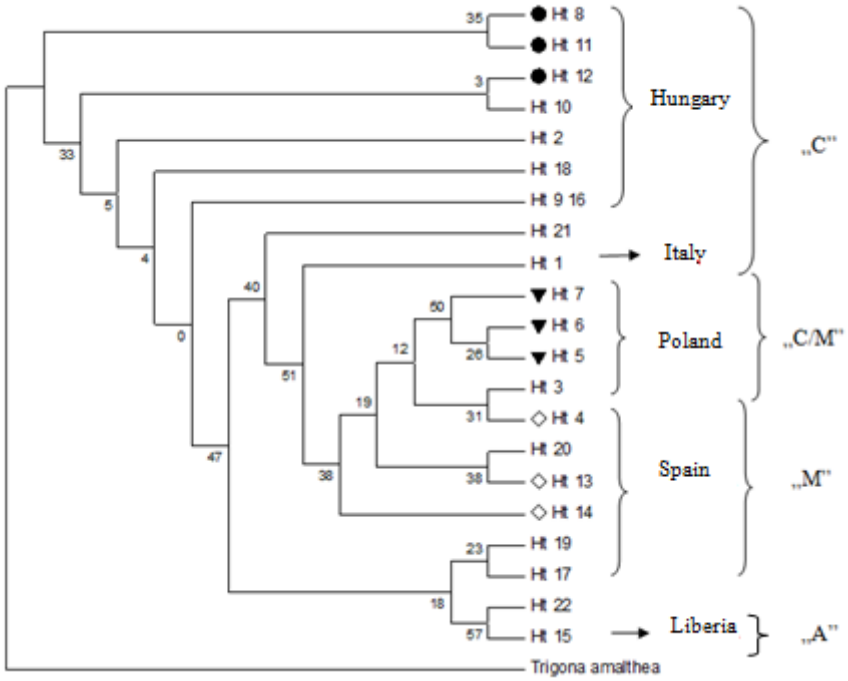


Figure 2: Maximum Likelihood phylogenetic tree, Hasegawa Kishino Yano plus Gamma model, number of Bootstrap runnings: 10.000
(●- new haplotype from Hungary, ▼- new haplotype from Poland, ◇- new haplotype from Spain)

Table 2: pairwise F_{ST} values among populations
ns- not significant *p<0.05, **p<0.01, ***p<0.001

	Hungary	**Italy**	**Bialowieza**	**Siedlice**	**Krakkow**	**Bydgoszcz**	**Wroclaw**	**Spain**	**Liberia**
Hungary	-	***	ns	***	ns	**	***	***	***
Italy	0.277	-	***	***	***	**	ns	**	ns
Bialowieza	0.061	0.105	-	***	***	***	***	***	***
Siedlice	0.042	0.111	-0.116	-	***	***	***	***	***
Krakkow	0.160	0.129	-0.081	-0.064	-	***	**	***	***
Bydgoszcz	0.870	0.521	0.666	0.678	0.657	-	ns	ns	ns
Wroclaw	0.883	0.510	0.758	0.771	0.725	0.145	-	ns	ns
Spain	0.788	0.357	0.523	0.537	0.518	0.418	0.333	-	ns
Liberia	0.938	0.822	0.956	0.957	0.924	0.814	0.908	0.641	-

Via the sequences we used, we detected nine new haplotypes that had not been in the NCBI database. The three haplotypes that are marked with black circles we identified as new (Ht 8, Ht 11 and Ht 12). The black triangles show the newly identified three Polish haplotypes (Ht 5, Ht 6 and Ht 7). The three new Spanish haplotypes (Ht 4, Ht 13 and Ht 14) are marked with blank rhombus. As expected the Spanish and Liberian (Ht 15) haplotypes are well differentiated. *Trigona amalthea*, a wild bee type that was used for control can be well distinguished from the domestic bees (Figure 2).

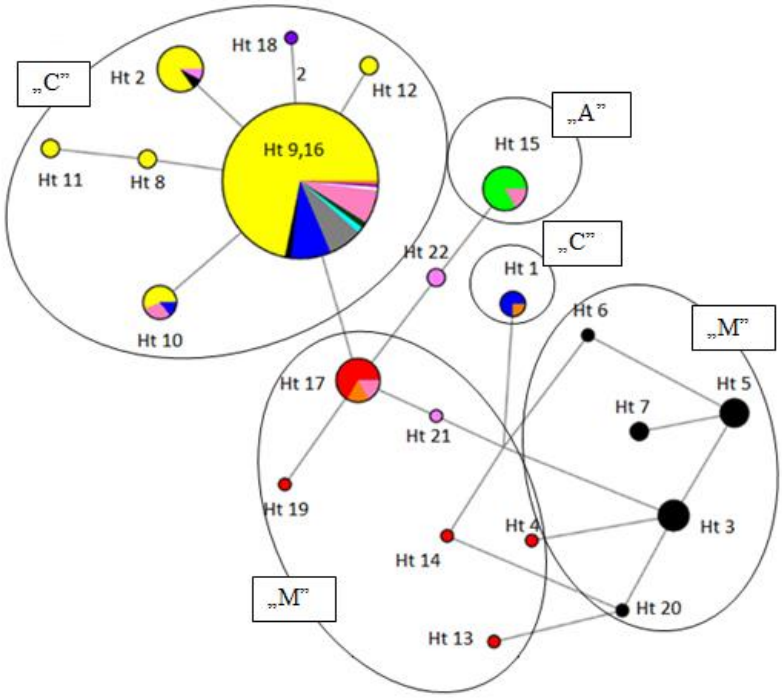


Figure 3: The relation of the haplotypes that was established among the Hungarian, foreign control and the 53 reference sequences, with the help of Median-Joining Network analyses

Colour code: yellow - *A. m. carnica*, dark blue - *A. m. ligustica*, black - *A. m. mellifera*, grey - Buckfast line, light green - *A. m. adansonii*, red - *A. m. iberica*, orange - *A. m. sicula*, violet - *A. m. anatolica*, light blue - *A. m. caucasica*, dark green - *A. m. adami*, white - *Bombus terrestris*, pink - sequence appearing in the NCBI gene bank without the exact naming of the species

On the Figure 3. Median-Joining Network analysis was performed. The size of the circles is proportional with the number of the haplotype individuals, the different colours show different subspecies. Next to the haplotypes we added the number of the haplotypes.

During the haplotype relationship studies in the Hungarian bee samples we identified that the Hungarian Carniolan subspecies belongs to one haplogroup. Within the haplogroup

there are six well distinguished haplotypes, as the Ht 16 melted into the Ht 9. Within the most common Ht 91.3% *A. m. mellifera*, 8.3% *A. m. ligustica* and 7.05% Buckfast line's individuals appeared. In addition to this, the presence of the black bee can be found in the Ht 2, as well.

3.2. Results of the microsatellite study

Altogether we detected 172 alleles in the 10 examined populations with using 9 microsatellite markers. The number of the alleles is between 10 and 31, with this the values of the expected and real heterozygosity and polymorphism have changed in upright proportions. We identified the least alleles on the A28 and A(B)24 locus, and the most on the A107 loci.

Based on the *F*-statistical results we can state that in the case of all of the examined locus there are a number of heterozygotes, and as a result the inbreeding factor's values are relatively low, so in the examined population the inbreeding is not typical.

The markers we used, except for the A28 marker have high polymorphism values (PIC average=0.635). The lowest heterozygosity value (*He*=0.407) and the lowest PIC value (PIC=0.36) were shown as a result of the A28 marker. Notwithstanding we identified the highest polymorphism index value (PIC=0.78) in the case of the A107 marker.

We verified that the real heterozygosity value of an average population is between 0.816 and 0.985, while the expected heterozygosity value was changing between 0.846 and 0.634. In each population we got high heterozygosity value, but the Liberian stock is the most significant (*Ho*=0.985±0.029). The high heterozygosity values we identified for the Hungarian apiaries show that we currently do not need to worry about inbreeding failure. The average allele numbers were 14.3±6.2 (Hungary) and 3.7±1.2 (Poland – Wroclaw). The unique allele proportion in the Hungarian bee population is 3.6% (Table 3).

The Table 4. show the examined populations' variance from the Hardy-Weinberg equilibrium. The variances were distinguished by three significance levels ($p<0.05$, $p<0.01$, $p<0.001$). Only the Hungarian population deviated on all locus from the Hardy-Weinberg equilibrium at $p<0.001$ significance level.

Table 3: The heterozygosity of the examined bee population
n = examined individual number, *Ap* = average allele number, *Np* = frequency of private alleles, *Ho*= real heterozygosity, *He* = expected heterozygosity

Populations	**Subspecies**	**n**	***Ap***	***Np* (%)**	***He***	***Ho***
Hungary	*A. m. carnica*	236	14.3±6.2	3.667±0.764	0.657±0.150	0.896±0.224
Liberia	*A. m. adansonii*	15	8.1±3.0	2.111±0.588	0.846±0.048	0.985±0.029
Spain	*A. m. iberica*	10	5.2±2.7	0.444±0.242	0.644±0.291	0.816±0.307
Bydgoszcz	*A. m. mellifera*	15	6.4±3.8	0.000±0.000	0.712±0.151	0.881±0.237
Krakkow	*A. m. carnica*	15	6.8±3.2	0.111±0.111	0.734±0.143	0.903±0.200
Bialowieza	*A. m. carnica*	15	7.4±2.6	0.222±0.147	0.747±0.129	0.822±0.270
Wroclaw	*A. m. mellifera*	6	3.7±1.2	0.000±0.000	0.709±0.103	0.907±0.188
Siedlice	*A. m. carnica*	15	6.1±2.2	0.111±0.111	0.756±0.098	0.888±0.309
Hungary	Buckfast line	10	4.3±1.2	0.222±0.222	0.644±0.217	0.843±0.296
Italy	*A. m. ligustica*	15	5.3±2.7	0.222±0.222	0.634±0.190	0.911±0.266
Mean (deviation)			**6.7±2.8**	**0.711±0.241**	**0.708±0.152**	**0.885±0.232**

Table 4: The diversity of the examined populations from the Hardy-Weinberg equilibrium
ns – not significant, *p<0.05, **p<0.01, ***p<0.001

	A113	A107	A28	A88	A14	A(B)24	A43	A35	A7
Hungary	***	***	***	***	***	***	***	***	***
Liberia		***						*	***
Spain		*			*	***			
Bydgoszcz		***		*			***	*	**
Krakkow		**		*			***	***	***
Bialowieza		***		*	***		**	***	**
Wroclaw								**	
Siedlice		**	**	*			***	***	***
Buckfast		**					**	**	***
Italy	*	*		*			***	***	***

We defined 53 unique alleles on the 9 loci in 29 populations, and 23 stock specific alleles in the Hungarian populations on 7 loci. For the purpose of gene sustaining it is especially important to reveal and protect the alleles that are rare or only exist in one population. In the Hungarian apiaries only on the A28 and A35 loci we did not identify special alleles. We detected the most unique alleles on the A107, A113 and A14 loci, therefore these markers are the most informative ones.

We got to similar results in the case of both the pairwise F_{ST} values and the Nei revised standard genetic divergence (*Ds*). On the Table 5. we marked in grey the not significant data. With the exception of the populations of Bydgoszcz and Wroclaw (p<0,01) in all other cases we determined significant results at p<0,001 level. The Liberian (0.216) and Spanish (0.291) populations were the farthest from the Hungarian populations. According to the examined microsatellite markers the Polish Bydgoszcz (0.167) and Wroclaw (0.136) populations showed the greatest divergence values from the Hungarian populations, greater than the Bialowieza (0.084), Krakkow (0.029) and Siedlice (0.045) populations. We can identify a genetic barrier among these populations, which is validated by the graph made with the Barrier program, the results of the main component analysis at population level and also the results of the mitochondrial DNA studies. Supposedly the *A. m. mellifera* species spread towards the western area of Poland and the *A. m. carnica* subspecies towards the eastern area. The greater divergence value of the black bee (Bydgoszcz, Wroclaw) from our domestic

Carniolan bee confirms that on the western part of Poland it is indeed the *A. m. mellifera* subspecies that is dominant, therefore their usage as control is reasonable. We got very similar results with comparing the outcome of the AMOVA test and pairwise F_{ST} values (Table 5, 6).

Table 5: Pairwise F_{ST} values (under the diagonal) and Nei-type revised standard genetic divergence (*Ds*) (above the diagonal)

	Hungary	**Liberia**	**Spain**	**Bydgoszcz**	**Krakkow**	**Bialowieza**	**Wroclaw**	**Siedlice**	**Buckfast**	**Italy**
Hungary	-	1.263	1.809	0.550	0.082	0.236	0.441	0.130	0.257	0.144
Liberia	0.216	-	0.958	1.021	1.095	0.941	1.039	1.030	1.455	1.384
Spain	0.291	0.157	-	0.341	0.886	0.632	0.412	0.797	1.249	1.558
Bydgoszcz	0.167	0.154	0.092	-	0.235	0.186	0.174	0.242	0.539	0.651
Krakkow	0.029	0.148	0.189	0.063	-	0.130	0.243	0.072	0.197	0.161
Bialowieza	0.084	0.132	0.146	0.047	0.025	-	0.082	0.062	0.173	0.196
Wroclaw	0.136	0.146	0.110	0.032	0.049	0.001	-	0.050	0.296	0.385
Siedlice	0.045	0.135	0.167	0.058	0.003	0.001	-0.007	-	0.176	0.151
Buckfast	0.097	0.205	0.273	0.153	0.057	0.051	0.095	0.054	-	0.164
Italy	0.060	0.212	0.298	0.183	0.053	0.066	0.126	0.051	0.064	-

Table 6: Results of the AMOVA test between the studied populations (1000 running)
ns – not significant, *p<0.05, **p<0.01, ***p<0.001

(%)	**Hungary**	**Liberia**	**Spain**	**Bydgoszcz**	**Krakkow**	**Bialowieza**	**Wroclaw**	**Siedlice**	**Buckfast**	**Italy**
Hungary	-	***	***	***	***	***	***	***	***	***
Liberia	34%	-	***	***	***	***	***	***	***	***
Spain	46%	27%	-	***	***	***	***	***	***	***
Bydgoszcz	27%	22%	27%	-	***	**	*	**	***	***
Krakkow	12%	21%	34%	12%	-	ns	**	ns	***	***
Bialowieza	16%	22%	30%	10%	2%	-	ns	ns	*	***
Wroclaw	30%	25%	36%	8%	10%	4%	-	ns	*	***
Siedlice	14%	21%	31%	9%	3%	2%	3%	-	*	**
Buckfast	22%	31%	49%	19%	10%	6%	13%	7%	-	***
Italy	18%	35%	49%	29%	12%	9%	30%	15%	18%	-

In the Table 7. the assignment study shows what percentage of the individuals truly fit into the hypothetical populations. We determined based on the results that the Hungarian population is fundamentally homogeneous (93.6%), although in small-scale some characteristic of foreign native genes can be observed. In our country we can identify characteristics of the Buckfast line (1.7%), the Italian bee (*A. m. ligustica*) (2.5%) and the black bee from Poland (*A. m. mellifera*) (2.1%). We can verify that the Liberian individuals really belong to the *A. m. adansonii* subspecies, and therefore are proved to be perfect controls. This is also true for the Spanish *A. m. iberica* subspecies.

Table 7: „Assignment test"

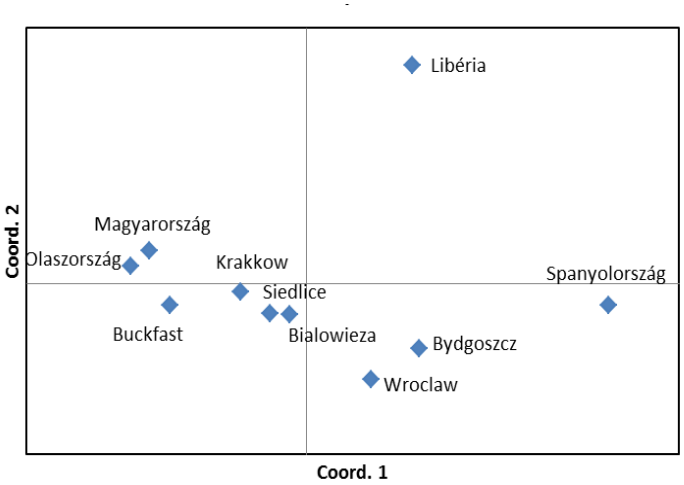
(%)	Hungary	Liberia	Spain	Poland	Buckfast	Italy
Hungary	93.6	0	0	2.1	1.7	2.5
Liberia	0	100	0	0	0	0
Spain	0	0	100	0	0	0
Poland	12.1	0	0	80.3	4.5	3
Buckfast	0	0	0	10	90	0
Italy	20	0	0	0	0	80

The Table 8. shows how many individuals truly belong to the assumed populations. It is well validated in the results of the divergence values that in the Hungarian population one can detect the presence of the gene stocks of the supposedly Carniolan types from Siedlice, Krakkow and Bialowieza. From Wroclaw only one individual of the *A. m. mellifera* subspecies is present. It can be established that the presence of the black bee in our country is negligable. In addition to this, in the Hungarian stock we could identify five Italian bee individuals and 3 belonging to the Buckfast line. In the table we highlighted in red the populations with Carniolan characteristics and in blue the apiaries with black bee dominance. It is shown in the table that the Polish population is widely heterogeneous, it is typical that the western area's bee population is *A. m. mellifera*, while on the eastern area the main species is mainly supposedly *A. m. carnica*. The majority individuals of the eastern apiaries of Poland have not faded into the Hungarian population, which presumably refers to the differences within the species.

Based on the Main component analysis results the African and Spanish populations is greatly divergent of the Hungarian apiaries.

Table 8: „Assignment test", results of the study at individual level
(Notations: IT-Italy, BU-Buckfast line, SI-Poland/Siedlice, WR-Poland/Wroclaw, BW-Poland/Bialowieza, KR-Poland/Krakkow, BY-Poland/Bydgoszcz, SP-Spain, AF-Liberia, HU-Hungary

(individual)	HU	AF	SP	BY	KR	BW	SI	WR	BU	IT	Number of studied bees (n)	Number of bees incorrectly assigned	Percentage of bees correctly assigned into their group (%)
Hungary	**201**	0	0	0	**8**	**4**	**14**	**1**	3	5	236	35	14.8
Liberia	0	15	0	0	0	0	0	0	0	0	15	0	0
Spain	0	0	10	0	0	0	0	0	0	0	10	0	0
Bydgoszcz	0	0	1	**11**	**2**	0	**1**	0	0	0	15	4	26.6
Krakkow	**3**	0	0	**1**	**5**	**2**	**4**	0	0	0	15	10	66.6
Bialowieza	0	0	0	**2**	**2**	**3**	**5**	**1**	1	1	15	12	80
Siedlice	**4**	0	0	**1**	**1**	**2**	**2**	**2**	2	1	15	13	86.6
Wroclaw	0	0	0	0	0	0	**1**	**5**	0	0	6	1	16.6
Buckfast	0	0	0	0	0	0	2	0	8	0	10	2	20
Italy	3	0	0	0	0	0	1	0	0	11	15	4	26.6



1. Figure 4: Main component analysis of the studied 10 bee populations

The Italian and the Buckfast populations are the closest to the Hungarian. In accordance with the former results the apiaries of Krakkow, Siedlice and Bialowieza are genetically relatively close to the Hungarian apiaries. So we verified again that the aforementioned populations can significantly carry Carniolan gene characteristics (Figure 4).

During the cluster analysis of the honey bee population (Figure 5) we identified that the Hungarian Carniolan Pannon form separated from the Liberian, Spanish and Polish Bydgoszcz populations already at the K=2 grouping. The Spanish *A. m. iberica* subspecies and the *A. m. mellifera* subspecies from Bydgoszcz are close to each other on the UPGMA phylogenetic tree, which is justified by their common "M" line of origin. After the K=3 grouping the Buckfast and the Italian populations separated from the other stocks. The closeness of the aforementioned two populations on the phylogenetic tree is also visible. Hereinafter (K=6) we can observe the separation of the Liberian *A. m. adansonii* subspecies from the Spanish *A. m. iberica* and the Bydgoszcz *A. m. mellifera* subspecies. During the following runnings the program could not differentiate between each population.

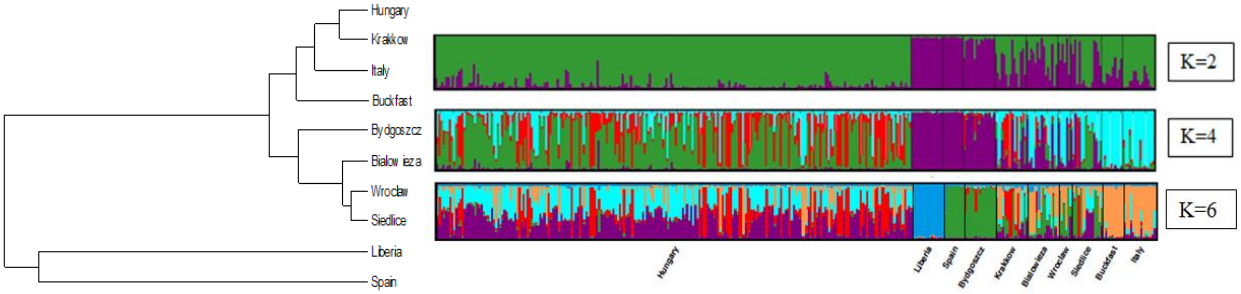


Figure 5: Cluster analysis of the studied honey bee populations (K= number of groups), Nei of corrected standard genetic divergence (*Ds*) based on the UPGMA phylogenetic tree

3.3. Results of the morphological study

During the morphological study of the Hungarian apiaries we measured 3 parameter values and the value of the K19 angle that is considered suitable as per the related literature.

In Table 9. we highlighted in bald the minimum and maximum values in the average bee families, and at individual level.

According to certain apiaries' average the cubital index falls between 3.06 and 2.51. Regarding the overall Hungarian bee population at individual level the smallest cubital index value was 1.53 while the greatest was 4.50.

At nationwide average the length of the proboscis was 6.6 mm, which value based on the average of the bee families was between 6.490 and 6.705 mm. At individual level we detected values between 4.48 and 7.00 mm for the proboscis length. The morphological standard used in Hungary defines the proboscis length of the Carniolan bee between 6,5 mm and 6,8 mm. We proved that based on this parameter and the bee families' average the Hungarian bee families belong to the Pannon form of the Carniolan subspecies. We defined small variance both on the basis of the cubital index values and proboscis length (Table 9).

We experienced the greatest variance at recording the parameters of the K19 angle, however the average value of the families was between 77.61 and 80.07.

Despite expected the results show that appearance of the Italian *A. m. ligustica* subspecies is not typical at the western areas close to Italy, but it is in the middle, eastern and southeast of the country.

Our results proved that 3 apiaries did not comply with the morphological standards because of the colours; and one queen breeding apiary because of the cubital index. Altogether the disqualification of 4 apiaries out of 16 would have been reasonable. In addition to this we verified that the characteristics of the Italian subspecies are in small scale present in the Hungarian honey bee population.

Table 9: The examined four morphological studies' parameter proportions and minimum and maximum values in the 16 Hungarian apiaries

Apiary	Cubital index	Min./Max.	Proboscis length (mm)	Min./Max.	Colour adequate (%)	K19	Min./Max.
1.	**2.51±0.43**	**1.53**/4.13	6.628±0.117	6.30/6.90	100	78.37±2.82	70.49/84.70
2.	2.80±0.39	1.93/**4.50**	6.600±0.110	6.30/6.80	40	**77.61±2.44**	71.81/82.57
3.	2.78±0.33	2.00/3.58	**6.705±0.111**	6.50/**7.00**	100	79.40±2.51	72.72/89.68
4.	2.74±0.43	1.81/3.81	6.538±0.127	6.10/6.80	20	78.24±2.85	71.16/**89.90**
5.	2.57±0.34	1.93/3.65	**6.490±0.182**	**4.48**/6.82	60	78.18±2.34	71.14/83.73
6.	2.61±0.31	1.96/3.59	6.640±0.118	6.30/**7.00**	60	**77.61±2.55**	71.28/83.30
7.	2.74±0.35	1.88/3.82	6.629±0.090	6.40/6.90	100	78.55±2.28	72.87/83.96
8.	2.63±0.34	1.82/3.53	6.587±0.119	6.30/6.88	100	79.10±2.61	73.91/84.70
9.	2.72±0.36	1.86/3.94	6.580±0.128	6.38/6.98	60	78.32±2.80	73.09/84.47
10.	2.72±0.33	1.71/3.47	6.604±0.120	6.28/6.90	80	78.32±2.80	73.09/84.47
11.	2.77±0.39	1.96/3.88	6.600±0.122	6.30/6.90	60	**80.07±2.98**	**70.06**/86.56
12.	2.84±0.49	2.00/4.40	6.596±0.110	6.30/6.80	80	77.91±2.30	71.78/82.85
13.	2.73±0.36	1.93/3.81	6.620±0.113	6.20/6.80	80	78.35±2.40	71.70/83.60
14.	2.76±0.36	1.93/4.19	6.576±0.096	6.30/6.90	0	78.95±2.42	71.89/85.10
15.	**3.06±0.37**	2.08/4.24	6.589±0.111	6.20/6.90	80	79.43±2.32	73.99/85.01
16.	2.72±0.36	1.94/3.80	6.625±0.099	6.40/6.90	80	78.59±2.28	72.79/83.40
Mean (deviation)	2.73±0.37	1.89/3.89	6.600±0.117	6.19/6.88	68.75	78.56±2.54	72.11/84.87

IV. NEW AND NOVEL OBSERVATIONS OF THE THESIS

1. Via the defined sequences of a section of the cytochrome-oxidase I mitochondrial region we detected seven haplotypes in the Hungarian honey bee populations (Ht 2, Ht 8, Ht 9, Ht 10, Ht 11, Ht 12 and Ht 16), of which the Ht 8, the Ht 11 and the Ht 12 appeared as new. In addition to this we identified Ht 4, Ht 13 and Ht 14 from Spain, and Ht 5, Ht 6 and Ht 7 from Poland. We proved that the dominant Ht 9 individuals in Hungary belong in 8.3% to *A. m. ligustica* subspecies, in 7.05% to the Buckfast line, and in 1.3% to *A. m. mellifera* subspecies. We established the dominance of Ht 9 in Central Europe, and that its frequency grows towards south.

2. With the help of nine polymorph microsatellite markers we proved that the Hungarian honey bee populations are nearly homogeneous (93.6%), in which the characteristics of the following species can be observed: 2.5% from *A. m. ligustica*, 2.1% from *A. m. mellifera* and 1.7% from the Buckfast line. We identified heterozygosity in the domestic Carniolan Pannon bees, therefore inbreeding is not typical. On 7 locus of the Hungarian bee population we detected 23 stock specific alleles. We proved genetic barriers between eastern and western apiaries of Poland. In addition, within the Carniolan subspecies we discovered the separation between the Hungarian and the Polish Carniolan populations.

3. The values of the morphological study parameters (colour, proboscis length, cubital index) used in Hungary for the Carniolan Pannon form have not significantly changed in the last 30 years. Of the apiaries we examined four did not comply with the Carniolan Pannon standards, therefore we proved the presence of the *A. m. ligustica* subspecies. The appearance of the Italian subspecies can be observed in the eastern part of our country.

4. After the comparison of the results of genetic and morphological studies, we concluded that the given results were not correlating to each other, however in all three apiaries both methods can be used to demonstrate the presence of the Italian subspecies.

V. PRACTICAL USABILITY OF THE RESULTS

With both molecular genetic and morphological studies we identified that the majority of the Hungarian bee populations is the Carniolan subspecies. In order to sustain the bee stock of the domestic Carniolan Pannon honey bee it is crucial to know the genetic structure of the populations. The rare alleles detected during our study, the small scale presence of foreign genetic effects in the domestic bee populations, and the information on the lack of inbreeding all contribute to form the classic breeding procedures, in this way our results can be used in practice, as well.

VI. PUBLICATIONS IN THE TOPIC OF THE THESIS

International peer-reviewed articles in English:

1. ***Zakar E.****-Jávor A.-Kusza Sz.:* 2014. Genetic bases of tolerance to *Varroa destructor* in honey bees. *Insectes Sociaux.* ISSN 0020-1812. (2012. IF: 1,331) (in press)

Peer-reviewed articles in Hungarian:

1. ***Péntek-Zakar E.****-Jávor A.-Kusza Sz.:* 2014. A mézhozam és a mézelő méh (*Apis mellifera* L.) morfológiai bélyegei közötti összefüggések vizsgálata. Irodalmi áttekintés. *Agrártudományi Közlemények.* ISSN 1587-1282. (in press)
2. ***Zakar E.****-Zajácz E.-Rácz T.-Oláh J.-Jávor A.-Kusza Sz.:* 2013. A hazai mézelő méh (*Apis mellifera* L.) populációk fajtajelleg vizsgálata. *Agrártudományi Közlemények.* 2013. 51: 59-63. ISSN 1587-1282.
3. ***Zakar E.****-Oláh J.-Jávor A.-Kusza Sz.:* 2012. Mikroszatellit markerek felhasználása a házi méhek (*Apis mellifera* L.) kutatásában. Irodalmi összefoglaló. *Állattenyésztés és Takarmányozás.* 61. 1: 37-46. ISSN 0230-1814.
4. ***Zakar E.****-Oláh J.-Jávor A.-Kusza Sz.:* 2012. A hazai mézelő méh (*Apis mellifera L.*) populációk genetikai távolságbecslésének vizsgálata. Előzetes közlemény. *Agrártudományi Közlemények.* 2012. 48: 61-64. ISSN 1587-1282.
5. ***Zakar E.****-Oláh J.-Jávor A.-Kusza Sz.:* 2011. Az ALH, HR78 és ND gének expressziójának változása atkafertőzöttség (*Varroa destructor* Oud.) hatására mézelő méhben (*Apis mellifera* L.). *Állattenyésztés és Takarmányozás.* 60. 1: 55-63. ISSN 0230-1814.

Publication for the public:

1. ***Zakar E.****-Oláh J.-Kusza Sz.:* 2013. A fajtajelleg vizsgálat története Világviszonylatban I. *Méhészet.* 61. 9: 10-11. ISSN 0465-6016.
2. ***Zakar E.****-Oláh J.-Kusza Sz.:* 2013. A fajtajelleg vizsgálat története Világviszonylatban II. *Méhészet.* 61. 10: 10-11. ISSN 0465-6016.
3. ***Péntek-Zakar E.****-Zajácz E.-Rácz T.-Oláh J.-Kusza Sz.:* 2013. Fajtajelleg vizsgálat eredményei Magyarországon. *Méhészet.* 62. 1: 6-7. ISSN 0465-6016.

4. ***Zakar E.***-*Oláh J.-Kusza Sz.:* 2010. Az ázsiai nagy méhatka. *Méhészet.* 58. 10: 3. ISSN 0465-6016.

Conferences:

Abstract proceedings in English:

1. ***Zakar E.***-*Zajácz E.-Rácz T.-Oláh J.-Jávor A.-Kusza Sz.:* 2013. Morphometric study of Hungarian honey bee (*Apis mellifera* L.) colonies. The 3rd Central European Section Meeting of the International Union for the Study of Social Insects. 2013. szeptember 14-18. Kolozsvár, Románia. 51.

Conference proceeding in Hungarian:

1. ***Zakar E.***-*Oláh J.-Jávor A.-Kusza Sz.:* 2011. Magyarországi házi méh populációk (*Apis mellifera L.*) genetikai távolságbecslése morfológiai adatok és mikroszatellit markerek alapján. I. AG-Biotech Debrecen Konferencia. Debrecen. 9.

Other publications:

1. *Tanács L.-Körmöczi L.-**Zakar E.:*** 2011. A Duna-Tisza közi homoki sztyepprétek vadméh-közösségének hosszú távú változásai. *Natura Somogyiensis.* 2011. 19: 201-222. ISSN 1587-1908.
2. *Tanács L.-Bereczkiné K. E.-Mészáros A.-**Zakar E.:*** 2011. Virághlátogató vadméh (*Hymenoptera, Apoidea*) közösség értékelése faunisztikai és szinbiológiai szempontok szerint Kisbugac-pusztán 2006-2008 között. *Agrár- és Vidékfejlesztési Szemle.* 6. 2: 222-233. ISSN 1788-5345.

Poster presentation in English:

1. *Szabó Gy.-Horváth R.-**Zakar E.**-Kozák L.-Lengyel Sz.:* 2010. The effect of grassland restoration on bee communities – a preliminary study in Hortobágy National Park, Hungary. Seventh International Congress of Hymenopterists in Kőszeg, Hungary.