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General Scientific Report

Project Number: PNII – Idei: 390/2007, cod ID: 852

Title of the project: Characterization of *Apis mellifera Carpatica* honeybee genetic potential using molecular techniques for biodiversity preservation

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2010

Introduction

The project entitled **Characterization of *Apis mellifera Carpatica* honeybee genetic potential using molecular techniques for biodiversity preservation** was conducted during Sept. 2007 - Sept. 2010 and aimed to identify ecotypes or varieties of honeybees in Transylvania.

The following key objectives were highlighted:

1. Scientific study of intra-specific diversity of *Apis mellifera* honeybees from Transylvania;
2. Sampling and preservation of biological material (*Apis mellifera Carpatica*) in Transylvania and establish working protocols;
3. Determination of morphometric measurements in *Apis mellifera Carpatica* variety;
4. Identification of molecular markers used in the testing methods to analyze the genetic variability of honeybees;
5. Testing method and selection of molecular marker;
6. Research on the genetic variability in honeybees;
7. Establishing correlations between morphometric characteristics and breed studied gene-pool;
8. Study of heterogeneity of populations studied and the degree of hybridization;
9. Developing technologies to increase the race *Apis mellifera Carpatica* variety to preserve local biodiversity;
10. Dissemination of the information.

Objective 1. Scientific study of intraspecific diversity of honeybees *Apis mellifera Carpatica* variety in Transylvanian Basin

This objective has been achieved in the first phase of the project by studying literature and identifying methods to highlight the genetic diversity of honeybees.

Objective 2. Sampling and preservation of biological material (*Apis mellifera Carpatica* variety) in Transylvania and establish the working protocols.

As it was mentioned in the 2007 report the exterior aspect of honeybees is determined by genetic and environmental factors. Exterior aspect of the honeybee is genetically determined as being constant, and it is characterizing each breed separately. Instead, it may change depending on seasonal variability, cells for brood size, quantity and quality of food for juveniles. Therefore were selected the criteria of honeybee preserving as follows:

- Stationary apiary located in isolated areas from migratory practice beekeeping.
- To avoid the phenomenon of hetero-sperm (taking into account the mating flight of the queen) there were chosen apiaries with minimum distance apart 50 km.
- The sampling period after the end of the swarming;
- There were chosen new honeybees (just emerged from the cells) just to be sure that those belong to a specific queen.
- There were chosen honeybees from new built honeycomb (of white to yellow coloring) to obtain accurate morphometric data (as the older combs are both developing area of bees is smaller and this negatively influences the morphometric results).
- A sample of honeybees contains about 100 individuals of young worker bees harvested from the same hive.
- There were chosen at least three locations per county.

Biological material: Bees collected according to criteria mentioned above were stored in Diethyl Ether (for carrying out morphometric measurements) and in 70% Ethanol for genetic analysis. Villages where sampling has been carried out are: Unirea (jud. Alba), Cut (jud. Alba), Cristian (jud. Sibiu), Bradu (jud. Sibiu), Vânători (jud. Mureș), Rupea (jud. Brasov), Rupea Gară (jud. Brașov), Făgăraș (jud. Brașov), Cristian (jud. Brașov), Arcuș (jud. Covasna), Zagoni (jud. Covasna), Poian (jud. Covasna), Păuleni Ciuc (jud. Harghita), Zetelaka (jud. Harghita), Suseni (jud. Harghita), Borsec (jud. Harghita), Râștolița (jud. Mureș), Petelea (jud. Mureș), Bălăureni (jud. Mureș), Cuci (jud. Mureș), Uriu (jud. Bistrița-Năsăud), Livezile (jud. Bistrița-Năsăud), Fiad (jud. Bistrița-Năsăud), Leordina (jud. Maramureș), Tisa (jud. Maramureș), Baia Sprie (jud. Maramureș), Medieșu Aurit (jud. Satu Mare) Doba (jud. Satu Mare), Petri (jud. Satu Mare), Săcueni (jud. Bihor), Camăr (jud. Sălaj), Românaș (jud. Sălaj), Bădești (jud. Cluj), Băișoara (jud. Cluj) (Fig. 1). In the county of Sibiu most beekeepers operate

Buckfast hybrid bee or pastoral practice, which is why only a single sample was collected (Cristian). Bees were kept at -20°C until the test.

For each apiary were collected cca. 100 honeybees, and DNA extractions were done from 10 bees for microsatellite analysis (3rd pair of legs) and 10 bees for analysis of mitochondrial DNA (thoracic muscles). Morphometric measurements were conducted on a total of 30 bees.

Chemicals: Sodium form Chelex 100 (Sigma), proteinase K (Promega), 2x PCR Master Mix (Fermentas or Promega), Forward Primer: B 124, A 7, A 24, A 28, A 35, A 107, A 113, A 88 (Eurogentec); Reverse Primer: B 124, A 7, A 24, A 28, A 35, A 107, A 113, A 88 (Eurogentec), agarose powder (Promega or Merck), 10 X TBE buffer (Lonza), MgCl₂ (25mM) (Promega), dNTP (10mM) (Promega), Diethyl Ether 74, 12 M g/mol (Merck); GoTaq 5 U/ml (Promega), ultra pure H₂O (Fermentas or Promega), Colour: Blue/Orange 6X Loading Dye (Promega) and 6x DNA Loading Dye (Fermentas); Ladder: 100 bp DNA Step Ladder (Promega) and Ruler Grene 1KB DNA Ladder (Fermentas), SYBR Safe DNA gel stain 10 x (Invitrogen).

Electronic devices and instruments used for morphometric measurements: dissecting kit and Olympus Stereomicroscope SZX 16, Olympus Camera: OLYMPUS SP-350, Light Source: OLYMPUS KL 2500 LCD; Quick MICRO PHOTO 2.2 software. Apparatus used for genetic analysis: PCR thermo-cycler Corbett Reserch, Eppendorf Mastercycler personal electrophoresis Line: - The current line EV 261 Consort; tank migration: Consort n.v. and Transiluminator: UVP, Spectrophotometer: Beckman Coulter DU 730.

Objective 3. Determination of morphometric measurements in *Apis mellifera* Carpatica variety

Method used:

1. Morphometric measurements

The morphometric measurements were performed according to methods described by Ruttner (1988) adapted. Head measurements (Figure 1) are: internal line between ocelli (POL), the line between compound eyes and ocelli (OOL), the side line of the eye (LOL), the size of labrum, length proboscis. Wing measurements were determined as follows: the 11 joint angles formed wing rib: A4, B4, D7, E9, G18, Q10, Q16, K19, L13, N23, O26, forewing length (FL), forewing width (FB), the length of radial cell (RC), the cubital index of cubital vein A (CV A) and cubital vein B (CV B). The following measurements were performed at hind wing (Figure 2): length of hind wing (LAP), the width of the hind wing (Laap), number of hooks (H). On the hind leg (Figure 3) is measured as follows: femur length (FE), tibia length (TI), metatarsus length (ML), metatarsus width (MT). The abdomen measurements (Figure 4) were determined as follows: the length of the 6th sternit (S 6), the width of the 6th sternit (LS 6), the length of the 5th sternit (S 5), the length of the 4th sternit (S 4). Other measurements were done at tergits.

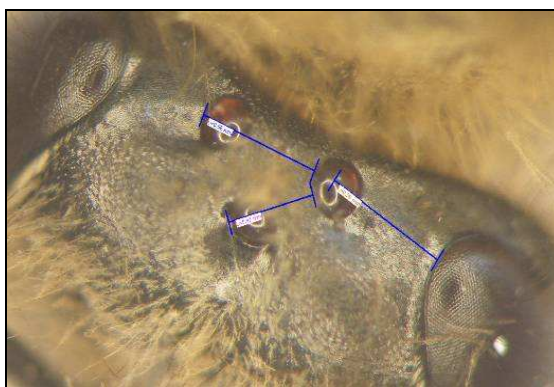


Figure 1- Compound eye and simple eyes (ocelli)

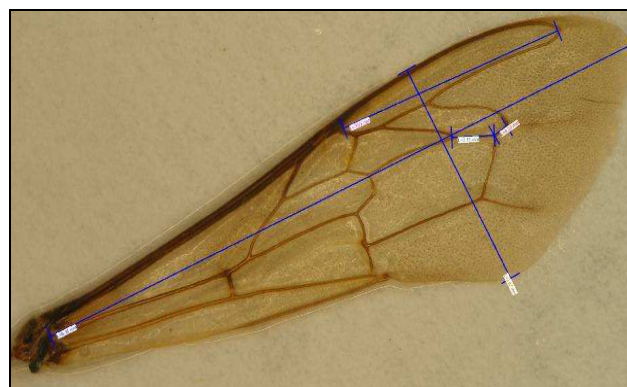


Figure 2 – Measurements of fore wing

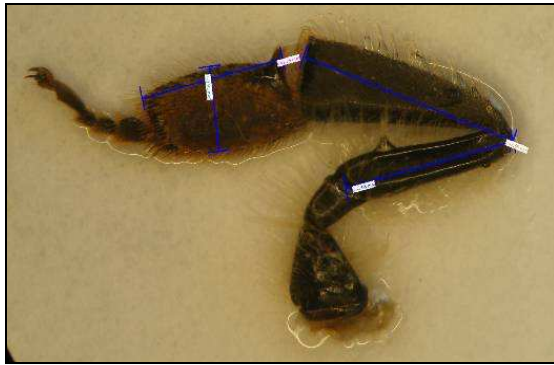


Figure 3 – Measurements of hind leg

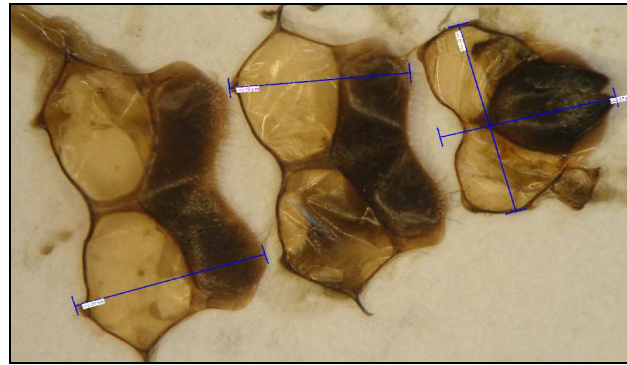


Figure 4 – Measurements of 6, 5 and 4 sternit

Statistical interpretation was performed using Excel, the program PAST 1.84.exe and Origin.

Results:

The analysis is taken within the ecotype of the Transylvanian Plain and Western Plain and Carpatica ecotype hilly area; in addition to these five ecotypes bees in Romania can be classified in different varieties: plain, hill and mountain. This adaptation can be foreseen if we consider the variety of environmental factors in this part of Transylvania, and if we take into account the laws of Bergmann, Allen and Glover on the adaptation of different species to environmental conditions. It could thus establish a correlation between environmental factors. To express the data more accurate two-step procedure was used for multivariate analysis: 1. factor analysis (principal component analysis - PCA - used to detect subgroups in a population in a given area). 2. Discriminatory analysis in stage (DA - to confirm and identify subgroups established character of the most discriminatory in order to calculate the distance between the center of the groups).

After statistical processing of the data obtained it can be concluded that there is no correlation between measured characteristics and environmental factors. The results of morphometric measurements do not discriminate against ecotypes identified during '70. Furthermore, no varieties of plain, hill or mountain can be observed. This is explained by mixing of ecotypes detected in more than 30 years ago because of intense practice of migratory beekeeping.

Objective 4. Identification of molecular markers used in the testing methods to analyze the genetic variability of bees

According to literature methods and materials were tested following molecular markers: E2 and H2, N1 and N2, 16 rDNA (ADNmt), CO I. From these primers E2 and H2 showed good results and reproducibility and therefore will be used in the following stages.

Objective 5. Molecular markers testing and the choice of working method

There were applied three different methods for the extraction of DNA: the classical method used for PCR phylogenetic analysis of molecular markers, Phenolic-chloroform method and Chelex method. As a conclusion the selected method for further analysis was Chelex method. In the methods of analysis used to test of genetic variability of *Apis mellifera* Carpatica variety from Transylvanian Basin using mitochondrial DNA, i.e. genomic DNA extraction, quantification of samples, PCR with specific primers, their migration in agarose gel, capturing image analysis and interpretation of data obtained and finally finding the degree of phylogenetic kinship between the populations analyzed.

DNA extraction (Chelex method) was done using an extraction protocol, using the following reagents: Chelex, proteinase K, 70% Ethanol, distilled water. As for the anatomical portion extraction thoracic muscles were used. Thermocycler test site was used with the following PCR program: 55 °C - 1 hour, 99 °C - 15 min., 37 °C - 1 min., 99 °C - 15 min.

The quantity and purity of DNA has been read using NanoDrop 1000 spectrophotometer. The results show small differences between the samples regarding the purity of samples analyzed (Table 1). The quantity and purity of DNA amplification can be considered good for them and analysis of mitochondrial DNA sequences. PCR reaction was based on the use of multiple "universal" sets of primers (which was already tested in several European research teams, which gave good results for the analysis of biodiversity of breed populations of *Apis mellifera*: *Apis mellifera lingustica*, *Apis*

mellifera caucasica, *Apis mellifera carnica*. Estimation of the number of base pairs is performed using the UVP system based on molecular size marker used.

Table 1

Quantity (ng/μl) and purity (260/280) of DNA extracted from thoracal muscles from *Apis mellifera carpatica* exploited in different counties from Transylvania

Anatomical region	N	sample COD	ng/ul	A260	A280	260/280	260/230
Thoracal muscles	15	I	291.46	5.989	3.049	1.96	0.66
Thoracal muscles	15	II	185.81	3.717	2.148	1.73	0.44
Thoracal muscles	15	III	342.73	7.415	5.932	1.25	0.57
Thoracal muscles	15	IV	382.61	27.652	27.169	1.02	0.49
Thoracal muscles	15	V	93.66	1.873	1.376	1.36	0.45
Thoracal muscles	15	VI	194.94	3.899	3.102	1.26	0.4
Thoracal muscles	15	VII	146.47	2.929	1.642	1.78	0.4
Thoracal muscles	15	VIII	210.37	24.207	21.189	1.14	0.49
Thoracal muscles	15	IX	161.29	3.226	2.265	1.42	0.43
Thoracal muscles	15	X	289.01	5.78	4.426	1.31	0.54
Thoracal muscles	15	XI	158.82	3.176	1.885	1.69	0.5
Thoracal muscles	15	XII	526.51	30.53	39.823	0.77	0.43

Samples migrated to test this protocol originated from Cluj county, city Băișoara, Bădești and Deus.

Objective 6. Research on the genetic variability in honeybees

- 1) Identify common genetic characteristics in the studied population and establish correlations between these bees

Apis mellifera Carpatica populations studied in terms of microsatellite variability data analysis (primer widely used for such analysis - see Table 2) allowed us to obtain results for the first time in Romania on the variability of SSR markers. SSR markers are a class of DNA markers involving a variable number of tandem repeats (minimum 10), 1-5 base pairs. Polialele of microsatellite loci are characterized by the same length expressed in base pairs. Fragments obtained after PCR, to amplify DNA sequences located between two single region linked by repeated in tandem, have a particularly high polymorphism. Because these fragments have accumulated mutations at a high rate during evolution, that these markers have a high polymorphism because of a large number of alleles at the same locus, useful for paternity analysis for mapping the genome. Because markers are part of co-dominant trees, they can be very useful in population genetics, especially in species in which variability is reduced. The microsatellite loci studied at populations of *Apis mellifera* were found to be very useful in characterizing the neighboring regions (Estoup *et al.*, 1993, 1994, Valdes *et al.*, 1993, Weber and Wrong, 1993). In the frame of analysis performed by our team, the microsatellite primers which showed a high polymorphism are:

Table 2

Microsatellite primers used for genetic analysis and alignment of their temperatures

Primer	Alignment temperature (°C)	Primer sequence
A107	F	5'-CCGTGGGAGGTTTATTGTCG-3'
	R	5'-GGTTCGTAACGGATGACACC-3'
A113	F	5'-CTCGAATCGTGGCGTCC-3'
	R	5'-CCTGTATTTTGAACCTCGC-3'
A24	F	5'-CACAAGTTCCAACAATGC-3'
	R	5'-CACATTGAGGATGAGCG-3'
A28	F	5'-GAAGAGCGTTGGTTGCAGG-3'
	R	5'-GCCGTTTCATGGTTACCAGG-3'
A35	F	5'-GTACACGGTTGCACGGTTG-3'
	R	5'-CTTCGATGGTCGTTGTACCC-3'

A7	F	58	5'-GTTAGTGCCTCCTCTTGC-3'
	R		5'-GCCTTCCTCTTTCATCTTCC-3'
A88	F	54	5'-CGAATTAACCGATTTGTCCG-3'
	R		5'-GATCGCAATTATTGAAGGAG-3'
B124	F	58	5'-GCAACAGGTCGGGTTAGAG-3'
	R		5'-CAGGATAGGGTAGGTAAGCAG-3'

Analysis of genetic variability were used eight microsatellite loci, coded as: B 124, A 7, A 24, A 28, A 35, A 107, A 113, A 88. Among these, only seven were used for statistical processing of data, because microsatellite A88 didn't amplify visible in all analyzed groups. After migration of PCR products, derived from amplification with primers specific for the seven microsatellite loci (B 124, A 7, A 24, A 28, A 35, A 107, A 113) the image of agarose gel was achieved by image capture system (UVP). Depending on the used molecular size marker (Ladder, 100 bp, Promega, USA), the amplified fragment size was estimated for each bee (10) and each sample (Cristian SB, Petri SM, Săcueni BH, Medieșu Aurit SM, Camăr SJ, Unirea AB, Cut AB, Cuci MS, Uriu, BN, Petelea MS, Baia Sprie MM, Vânători MS, Tisa MM, Zimbor MS, Finiș SJ, Făgăraș BV, Livezile BN, Rupea BV, Bădești CJ, Fiad BN, Arcuș CV, Băișoara CJ, Bradu SB, Zetea HR, Suseni HR, Borsec HR, Poian CV, Zagoni CV).

The next step (after estimating the size of amplified fragments in base pairs) was to give numerical codes to identified populations. In analyzed samples 29 subpopulations were identified. These genotypes obtained as a result of bands size estimation after gel migration are clearly differentiated in three different subgroups as shown in Figure 5. Following figure shows that the sample taken from the city Cristian (sample number 3), Buckfast hybrid is completely apart from the rest of the total samples.

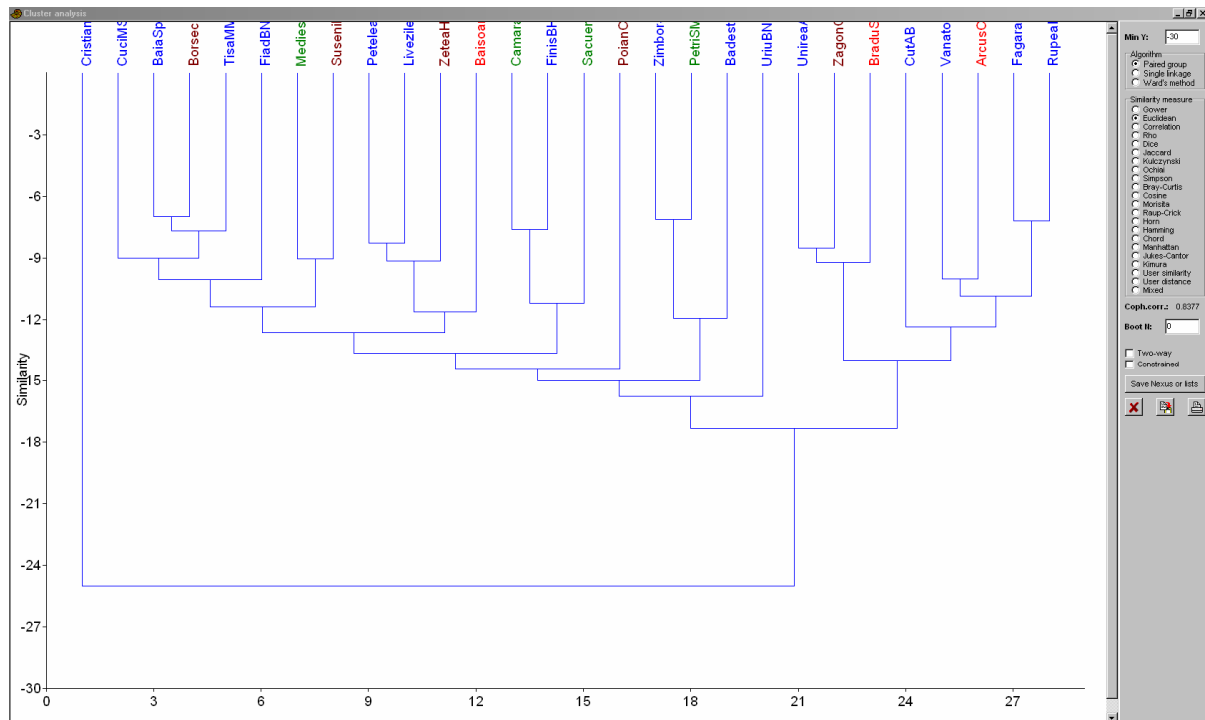


Figure 5 – Grouping of the genotypes (PAST ed. 1,85)

Each microsatellite has identified two or more genotypes, according to the fragment of recognition. These genotypes along with the identification marker are presented in Table 3.

The genotypes identified according to analysed SSR primer

Microsatellite	Genotype
B 124	4, 5, 8, 10, 11, 12
A 7	1, 7, 14, 15, 16
A 24	2, 25
A 28	3, 24
A 35	17, 27
A 107	18, 19, 20, 21, 22, 23, 26
A 113	6, 9, 11, 13, 28, 29

Factorial analysis of the different microsatellite loci presents results that complement each other. However, there is a genotype (adnoted with number 12 - Cristian city - SB), which has the most different genetic characteristics from other samples. The length and the number of amplified alleles are different according to the analyzed locus.

2) Identification of the gene loci that are specific to race *Apis mellifera* *Carpatica*

To identify the sites which provide distinct information about *Apis mellifera* *Carpatica* ADNmt analysis were performed, with the help of two sets of primers, used to analyse several populations of *Apis mellifera* from 1993 to present: primers E2 and H2 (Garnery *et al.*, 1993) and N1 and N2 (Moritz *et al.*, 2006). Study literature was needed for the amplified region with the afore-mentioned primers. In the case of primer set E2 (5' 'GGCAGAATAAGTGCATTG 3'-Forward) and H2 (5' CAATATCATTGATGACC 3'-reverse) there was noticed that these amplify the gene tRNA^{Leu} and the second subunit of COII gene (Cytochrome Oxidase II) with the length about 572 bp (GenBank: M23409.1, Locus AMFMTCOX) - Figure 6.

GenBank: M23409.1

LOCUS AMFMTCOX 2950 bp DNA linear INV 23-MAR-2000

DEFINITION *Apis mellifera* tRNA-Trp gene, complete sequence; cytochrome-c oxidase chain I gene, complete cds; tRNA-Leu gene, complete sequence; cytochrome-c oxidase chain II gene, complete cds; and tRNA-Asp and tRNA-Lys genes, complete sequence; mitochondrial genes for mitochondrial products.

tRNA 1820..1889

/product="tRNA-Leu"

5' **ggca gaataagtc attg 3'** primerul E2

1801 aatcaat ttt aattaaatt taat**ggca gaataagtc attg**aactta agattcaaat
 1861 ataaagtatt tttaaacttt tatta~~aa~~aatt tcccactta attcatatta atttaaaaat
 1921 aaattaata caatttttaa taaaataaat aattaat tttttatata tgaattttaa
 1981 attcaatcct aaagatttaa tctttttatt aaaattaata aattaatata aaataaaaca
 2041 aatataaca gaatatattt attaaaattt aatttattaa aatttcaca tgatttatat
 2101 ttatattca agaatcaaat tcatattatg ctgataattt aatttcattt cataatatag
 2161 ttataataat tattattata attcaacat taactgtata tattatttta gattattta
 2221 taaacaaatt ctcaaattta tttttattaa aaaatcataa tattgaaatt attgaaaca
 2281 ttattccaat tattattcta ttaattattt gttttccatc attaaaaatt ttattattaa
 2341 ttgatgaaat tgtaaactct ttttttcaa ttaatcaat **tggtcatcaa tgatattg**at
 2401 catatgaata tccagaattt aataatattg aatttgattc atatatacta aattataata

primerul H2 5' **caatcattgatgacc 3'**

Figure 6 – Sequences amplified with primer set E2 and H2 (Garnery *et al.*, 1993)

The fragment was amplified even for the samples of the present study, and its length was slightly different (approximately 580 bp - assessment in 1.5% agarose gel). This is a first indication that genetic material - *Apis mellifera* *Carpatica* variety is different from bees *Apis mellifera* *ligustica* for which the entire mitochondrial DNA has already been sequenced (Crozier and Crozier, 1993). Then the amplified fragment was "cut" with restriction enzyme *DraI* (Promega), which theoretically fragments the amplified segment into four smaller segments: two of 40-45 bp in length, one with a length of about

65 bp, one more about 420 bp long (according to the theoretical study of restricted loci studied in *Apis mellifera ligustica*, GenBank, access number: M23409.1).

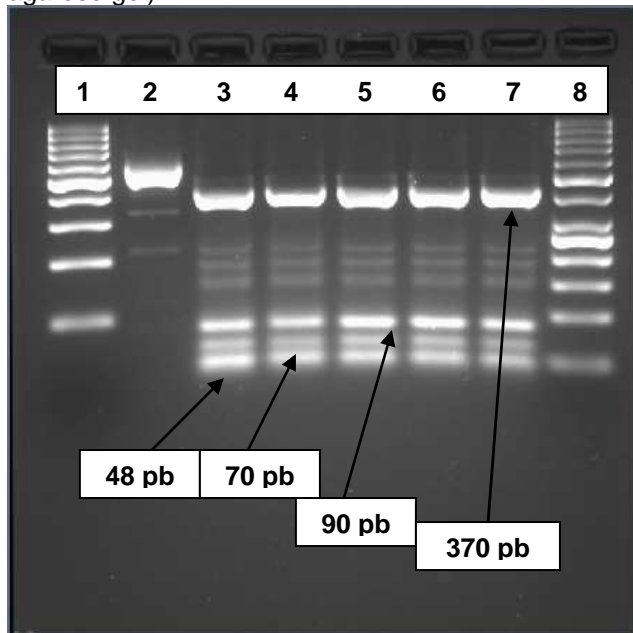
Regarding this specific fragment in analysed samples there were obtained fragments of different lengths from those of the bee *Apis mellifera ligustica*. For the bees from the 35 locations, it was found that there is no possibility of differentiation of distinct haplotypes. The samples comparison after microsatellite loci analysis resulted in differences between them of 100% (i.e. samples of bees from the area Cristian - Sibiu county, which are highly differentiated from other samples) from all other samples. With the restriction enzyme *DraI* of 580 bp fragment obtained, it did not present a different haplotype (Figure 7). This fragment thus does not provide the ability to differentiate *Apis mellifera* Carpatica from Buckfast hybrid using the restriction enzyme *DraI*. There were three restriction loci revealed by dividing the 580 bp fragment into 4 segments as follows:

- a segment of approximately 48 bp;
- a segment about 70 bp;
- a segment about 90 bp;
- a segment about 370 bp (Figure 7).

Previous studies on this specific fragment presented obvious differences between other species and subspecies of bees. Thus, *Apis cerana* bee populations in the Philippines, De La Rua *et al.* (2000) identified four haplotypes Ce1, Ce2, Ce3, Ce4, generally depending on islands where bees were collected. The fragments obtained were between 10 and 216 bp. Garnery *et al.* (1993) found 21 haplotypes due to this fragment, in a number of 302 beecolonies collected from an extensive area: Scandinavia, England, France, Spain, Sicily, Algeria, Morocco, South Africa, belonging to evolutionary lines A, C and M. By studying the same amplified region and the same restriction enzyme El-Niweiri and Moritz (2008) have found 7 haplotypes strains evolutionary, A and C: A1 (47 bp, 108 bp, 483 bp), A4 (47 bp, 108 bp, 193 bp, 483 bp), A8 (47 bp, 591 bp), A13 (47 bp, 310 bp, 483 bp), O1 (47 bp, 67 bp, 108 bp, 420 bp), O1' (47 bp, 67 bp, 67 bp, 108 bp, 129 bp, 420 bp), C2 (47 bp, 64 bp; 420 bp) in bees *Apis mellifera* exploited in Sudan (*Apis mellifera adansonii*, *Apis mellifera scutellata*, *Apis mellifera lamarckii* *Apis mellifera syriaca*). None of them match the fragment discovered by us, which belongs to the evolutionary line C.

Haplotype is as follows: 48 bp, 70 bp, 90 bp, 370 bp (the exact number of base pairs of each segment will be performed by sequencing).

Figure 7 - Electrophoretic profile of the samples restricted with enzyme *DraI* (3.5% agarose gel)



Line 1: molecular marker of 100 bp length (Fermentas);
 Line 2: DNA sample amplified with primers E2 and H2 unrestricted;
 Line 3: Sample from Unirea (MS) restricted with the enzyme *DraI*;
 Line 4: Sample from Cristian (SB) restricted with the enzyme *DraI*;
 Line 5: Sample from Bradu migratory area (SB) restricted with the enzyme *DraI*;
 Line 6: Sample from Vânători (MS) restricted with the enzyme *DraI*;
 Line 7: Sample from Rupea (BV) restricted with the enzyme *DraI*;
 Line 8: Marker molecular length of 50 bp (Fermentas).

Based on amplified fragment length analysis with primers E2 and H2 the biological material of studied apiaries can be categorized. Thus, the length of approximately 580 bp of the fragment allows us to frame those bees in *Apis mellifera carnica*, evolutionary line C, Carpatica ecotype (bees akin to

bee populations in South-Eastern Europe). After electrophoretic migration of amplified PCR products with primers N1 and N2 were revealed fragments of 790 bp in all studied populations.

3) *The study of molecular fingerprint to Apis mellifera Carpatica variety*

To obtain information on genetic variability of *Apis mellifera* exploited in Transylvania, our team has carried out complex analysis based on different experimental approaches: microsatellite loci, respectively ADNmt intergenic sequences. Based on these results the frequency of certain genotypes could be graphically presented.

Analysis of microsatellite loci. There were identified for each microsatellite marker specific allele of different sizes and specific numbers. There is therefore the possibility to use them as specific loci of Romanian honeybees. The loci detected by B124 microsatellite were alleles in homozygous state which form three distinct genotypes, and the size of amplified fragments is as follows: 190, 200 and 210 bp. After analyzing the A7 microsatellite locus were detected five different size genotypes (with fragments of 110 bp in the homozygous state, respectively 110-170 bp, 110-150 bp, 110-120 and 100-150 bp in the heterozygous state). After estimating the size of analyzed microsatellite DNA fragments with specific primer A107 there were eight genotypes of different alleles detected. These alleles occur in homozygous state in sizes of 180 bp, 160 bp and 100 base pairs and in heterozygous condition 180-200 bp, 160-180 bp, 180-220bp and 160-220 base pairs. The microsatellite A113 generated four genotypes at different alleles, two in homozygous state of 170 bp and 210 bp, and two heterozygous state of 170-210 bp and 210-250 bp. The microsatellite A28 allele has only two allele types, one homozygous and one heterozygous 150 bp 140-160 bp. The microsatellite A24 also has two allele types, a homozygous of 105 bp and a heterozygous of 90-110 bp. The microsatellite A35 is still with the two two allele types, a homozygous 110 bp and a heterozygous of 80-120 bp.

Analysis of mitochondrial DNA. There was possible to identify a new haplotype specific to studied populations using restriction enzyme *DraI* of the fragment amplified by E2 and H2 primers. Restrictive fragments are different from those obtained from other populations European bees. The sites of identified restriction loci were identified quite accurately (Figure 8), the next step being the sequencing of the entire fragment.

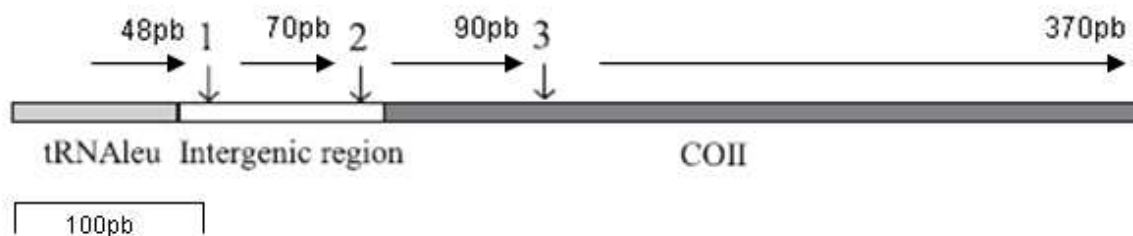


Figure 8 – Identifying sites of the restriction enzyme *DraI* in bee populations from Transylvania

4) *Statistical interpretation of results; certain characters degree of dispersion in the studied breed and varieties.*

Microsatellite loci analysis. The results of applying the Kruskal-Wallis test presents the differences between various samples of bees. Significant differences were identified between samples compared with the sample from Cristian SB. Thus, the difference between Cristian SB and Mediașu Aurit SM is $p = 0.00573$, Camăr SJ $p = 0.00873$, Cuci MS $p = 0.00397$, Tisa MM $P = 0.00655$, Fagaras BV $p = 0.00573$, Zetea HR $p = 0.00299$, $p = 0.00261$ Poian CV.

Analysis of mitochondrial DNA. Because fragments "cut" by the *DraI* enzyme are identical in all samples, the statistical interpretation is null for this marker.

Objective 7. Correlations between morphometric characteristics and gene pool of the studied breed

1). *Highlighting the genetic and phenotypic correlations in relation to selection based on production performance of the analyzed populations*

Phenotypically, honeybees are a relatively homogeneous population. Cristian sample is the only exception that has different colour characters on pigmentation in tergite 3. It is also possible to distinguish a single feature analysis that influences body size of bees, namely altitude. The body size is significantly correlated ($p = 0.04$) with altitude. In Figure 9 is very well observed clustering bees according to latitude and altitude (Bergmann's Law). The honeybees selected by brown colour belong from high altitude places, and the cluster surrounded with orange originates from hill and plateau.

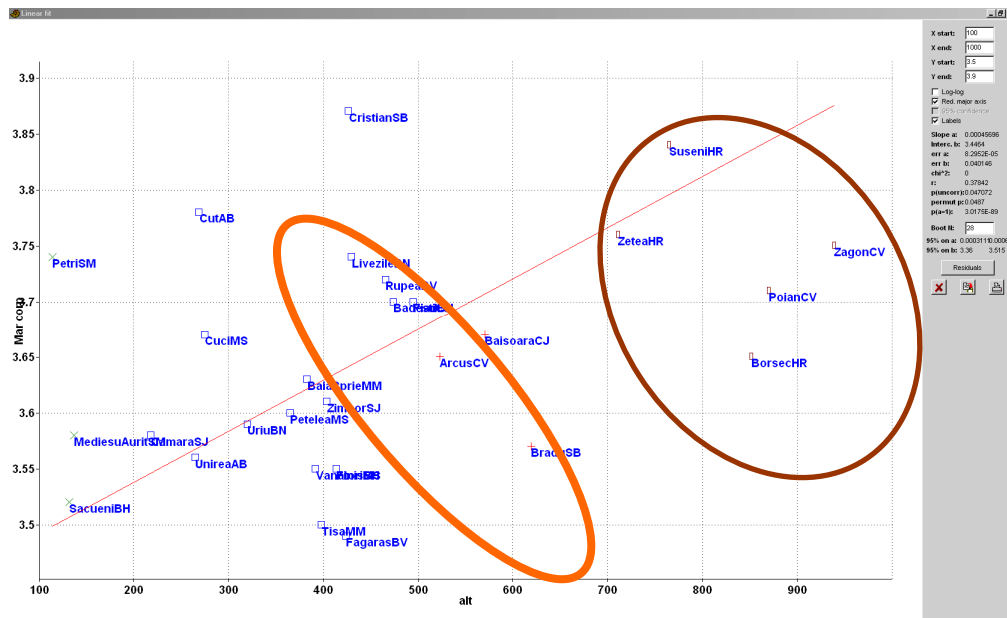


Figure 9 – Grouping of bees due to altitude (PAST ed.1,85)

Morphometric relative homogeneity is explained largely by practicing migratory beekeeping, but also by the fact that differences were probably quite weak in the past highlighted.

2) The nomination of the selection criteria taking into account the maintenance of biodiversity and genetic characteristics of breeds.

The present study observes the principles of an international program to maintain the biodiversity of species as a valuable source of genes is required to be preserved and protected especially. In this context, biological characteristics like adaptability, resistance to specific climatic conditions of a certain area, level and nature of diseases place *Apis mellifera carpatica* in a larger European and even worldwide framework. The main problem for biodiversity maintaining seems to be Colony Collapse Disorder (CCD). The causes are not fully identified, so is necessary to create a program to maintain the genes with increased strength and adaptability. It is therefore necessary that besides the classical criteria for the selection of bees (production, prolific queen, the intensity of bee flight during bad weather and harvesting, longevity, how to submit your honeybee aggressiveness, susceptibility to swarming, winter hardiness, disease resistance), to add genetic selection criteria (usage of genotyped queen and drones: different characters that confer resistance to environmental conditions of exploitation, disease with genetic resistance to parasites and viruses etc.).

Objective 8. Determination of heterogeneity of populations studied and the degree of hybridization

1) Analysis of heterogeneity in most populations

The studied genotypes observed ecotype homogeneous and characteristic are: Petri SM, Săcueni BH, Camăr SJ, Uriu, BN, Tisa MM, Zimbor MS, Finiș SJ, Bădești CJ, Fiad BN, Arcuș CV, Băișoara CJ, Brașu SB, Zagoni CV. Only 13 samples out of 35 were found to belong to local native stock (SNL) and not mixed with Buckfast hybrid.

Samples found to be mixed with Buckfast hybrid are the following: Cristian SB (90%), Medieșu Aurit SM (20%), Unirea AB (30%), Cut AB (20%), Cuci MS (30%), Petrele MS (10%), Baia Sprie MM (35%), Vânători MS (50%), Făgăraș BV (20%), Livezile BN (20%), Rupea BV (10%), Zetea HR (30%), Suseni HR (30%), Borsec HR (20%), Poian CV (60%). The characteristic genotypes of Buckfast hybrid were noticed to 15 samples. Otherwise, the studied bees belong to *Apis mellifera carpatica*

subspecie, a different population from other neighbouring populations, with its own phenotypic and genotypic characters.

2) *Identify the influx of foreign genes by quantifying mitochondrial DNA analysis*

The specific mitochondrial DNA markers used in the present project (Garnery *et al.*, 1993, Moritz *et al.*, 2006), can not make a differentiation of honeybee populations bred in Transylvania. The influx of foreign genes could be achieved only by sequencing the amplified fragments, and possibly use of other sets of primers that amplify other regions of mitochondrial DNA. But a new haplotype was found after restriction enzyme Dral fragment amplified with the primer set E2 and H2.

3) *Preparation of a map of different genotypes distribution in the studied population*

This action led to draw up a map of the territorial distribution of genotypes obtained by microsatellite primers used and the percentage of Buckfast hybrid hybridization.

Objective 9. Development of breeding technologies for *Apis mellifera Carpatica* variety in order to preserve local biodiversity

Courses were organized for beekeepers about:

- growth and replacement of queens belonging to native biological material in order to preserve local biodiversity;
- the artificial insemination of queens with selected drones for genetic transmission of certain production characteristics;
- selection of hive type according to beekeeper's production criteria;
- the type of treatment applied in prevention of bee diseases.

Objective 10. Dissemination of project results

The results have been promoted and disseminated through Web pages, scientific articles and courses for beekeepers. During this project were published 6 articles (2 ISI articles: the answer of editorial board is still expected, three articles in the database B +).