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SERICULTURE



Stage report

Stage III, 15 septembrie 2009

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

Director of project: Prof. Dr. Liviu Al. Mărghițaș
Project Number: PNII – Idei: 390/2007, cod ID: 852

- Cluj-Napoca, September 2009

The third stage of the project was evolved between 1st nov 2008 and 15th sept 2009.

The experimental design include three main objectives:

1. Research on the genetic variability of honeybees;
2. Establishing correlations between morphometric characteristics and gene pool of *Apis mellifera* Carpatica variety;
3. Determination of heterogeneity of populations studied and the degree of hybridization.

Each scientific objective includes its own activities. Samples were collected from 12 Transylvanian counties, from selected stationary and migratory apiaries (Figure 1). For each apiary were collected 100 bees, and DNA extractions were performed from 10 bees for microsatellite analysis (feet) and 10 bees for analysis of mitochondrial DNA (thoracic muscles). Morphometric measurements were conducted on a total of 30 bees.

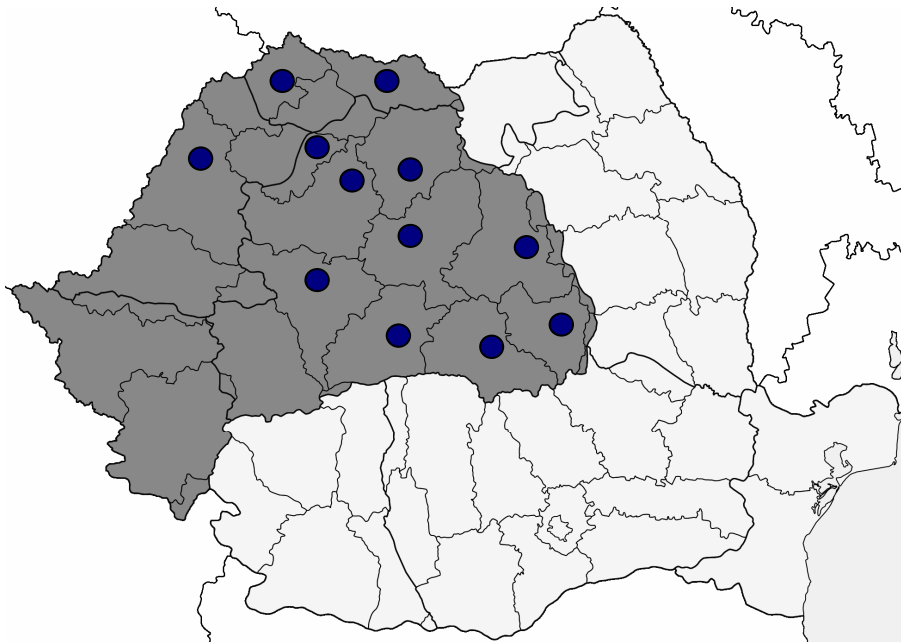


Figure 1 - Territorial distribution of the samples analyzed

First objective had 4 activities:

- 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 1) 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), of 1-5 base pairs. Poli-allele of microsatellite loci are characterized by the same length expressed in base pairs. Fragments obtained after PCR 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 or genome mapping. Because markers are part of co-dominant markers, they can be very useful in population genetics, especially in species in which alocizimic variability is reduced. The populations of *Apis mellifera* by studies in which microsatellite loci were found to be abundant and very useful in characterizing the neighboring regions (Estoup *et al.*, 1993, 1994; Valdes *et al.*, 1993; Weber and Wrong, 1993). In analysis performed so far the microsatellite primers which showed a high polymorphism are:

Table 1

Microsatellite primers used for genetic analysis and alignment of their temperatures

Primer		Annealing temperature (°C)	Primer sequence
A107	F	60	5'-CCGTGGGAGGTTTATTGTCG-3'
	R		5'-GGTTCGTAACGGATGACACC-3'
A113	F	54	5'-CTCGAATCGTGGCGTCC-3'
	R		5'-CCTGTATTTTGCAACCTCGC-3'
A24	F	54	5'-CACAAGTTCCAACAATGC-3'
	R		5'-CACATTGAGGATGAGCG-3'
A28	F	60	5'-GAAGAGCGTTGGTTGCAGG-3'
	R		5'-GCCGTTTCATGGTTACCAGG-3'
A35	F	60	5'-GTACACGGTTGCACGGTTG-3'
	R		5'-CTTCGATGGTCGTTGTACCC-3'
A7	F	58	5'-GTTAGTGCCCTCCTCTTGC-3'
	R		5'-GCCTTCCTCTTTCATCTTCC-3'
A88	F	54	5'-CGAATTAACCGATTTGTCG-3'
	R		5'-GATCGCAATTATTGAAGGAG-3'
B124	F	58	5'-GCAACAGGTCGGGTTAGAG-3'
	R		5'-CAGGATAGGGTAGGTAAGCAG-3'

For analysis of genetic variability eight microsatellite loci were used annotated as follows: 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 did not 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 captured by UVP system. Depending on the size of molecular marker used (Ladder, 100 bp, Promega, USA), the size of the amplified fragment was estimated for each honeybee (10 honeybees per sample) and for 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 2. 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.

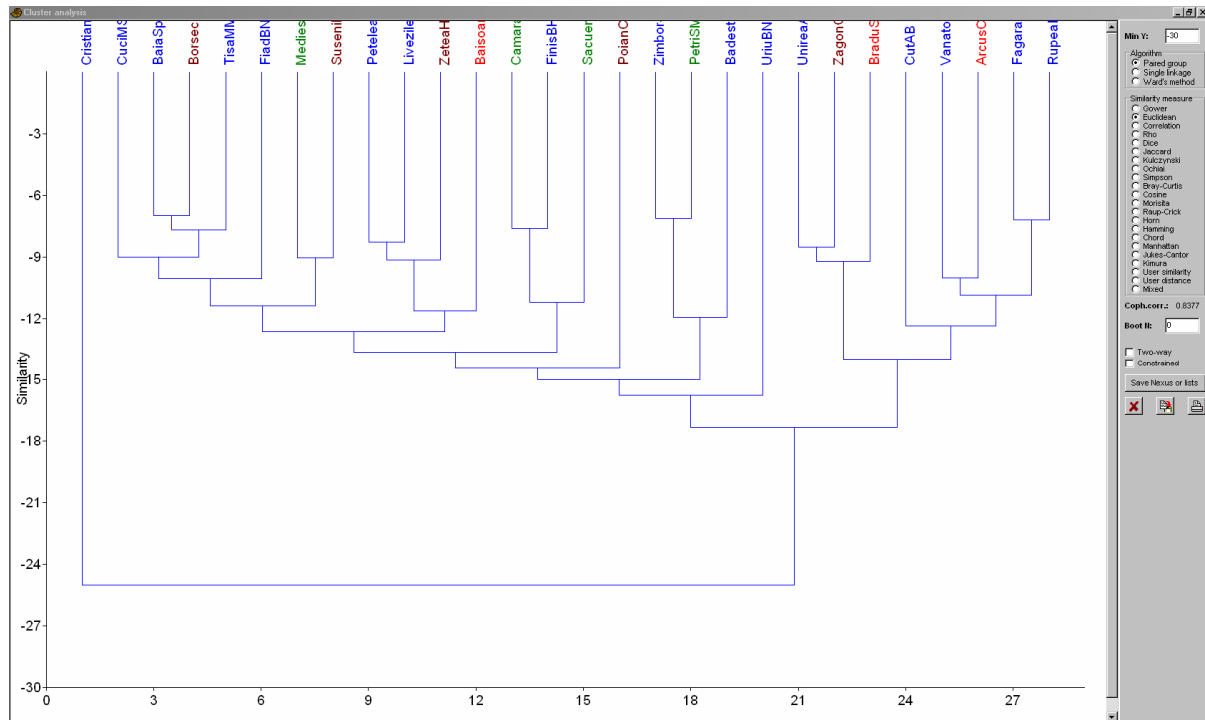


Figure 2 – Grouping of the genotypes (PAST ed. 1,85)

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 2.

Table 2

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 3.

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"

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                    5' ggca gaataagtgc attg 3' primerul E2
1801 aatcaat tttt aattaaat ttaataatggca gaataagtgc attgaactta agattcaaat
1861 ataaagtatt tttaaacttt tattaaat tccccactta attcatatta atttaaaaat
1921 aaattaataa caat ttttttaa taaaataaat aattaat ttttttataat tgaat ttttaa
1981 attcaatctt aaagat ttttaa tctttttatt aaaattaata aattaatata aaataaaaaca
2041 aaatataaca gaatatattt attaaaat taaattattaa aatttccaca tgatttatat
2101 ttatat ttttca agaatcaaat tcatattatg ctgataat tttttcattt cataatatag
2161 ttataataat tattattata atttcaacat taactgtata tattatttta gatttattta
2221 taaacaaatt ctcaaattta tttttattaa aaaatcataa tattgaaatt atttgaacaa
2281 ttattccaat tattattcta ttaattattt gttttccatc attaaaaatt ttatat ttttaa
2341 ttgatgaaat tgtaaactct tttttttcaa ttaaatcaat tggcatcaa/tgatattgat
2401 catatgaata tccagaat ttaataat ttaattgattc atatataacta aattataata

                    primerul H2 5'caatcattgatgacc 3'

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Figure 3 – 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:

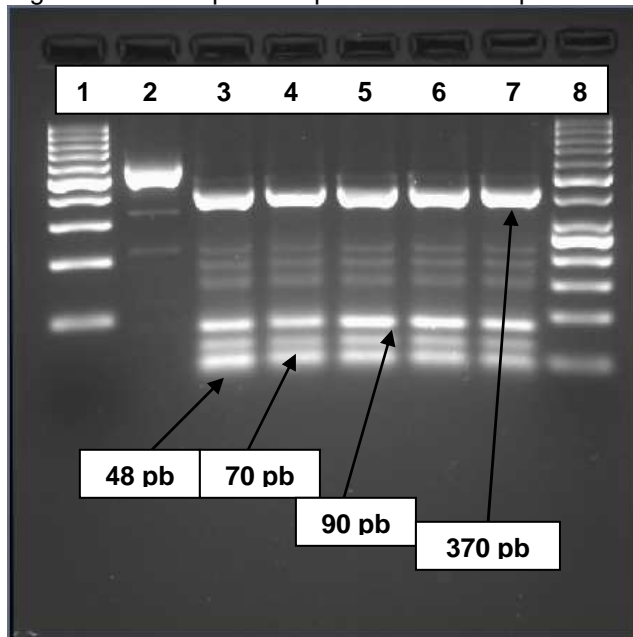
- a segment of approximately 48 bp;
- a segment about 70 bp;
- a segment about 90 bp;
- a segment about 370 bp (Figure 4).

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 4 - 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 5), the next step being the sequencing of the entire fragment.

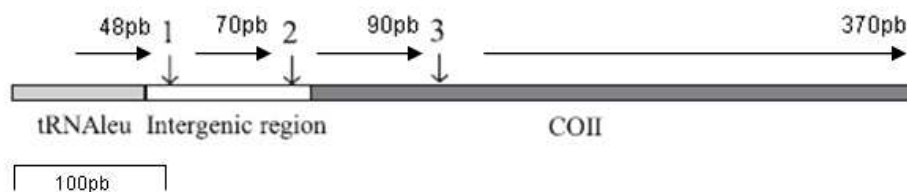


Figure 5 – Identifying sites of the restriction enzyme *DraI* in bee populations from Transylvania

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 2 has 2 activities:

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

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 6 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.

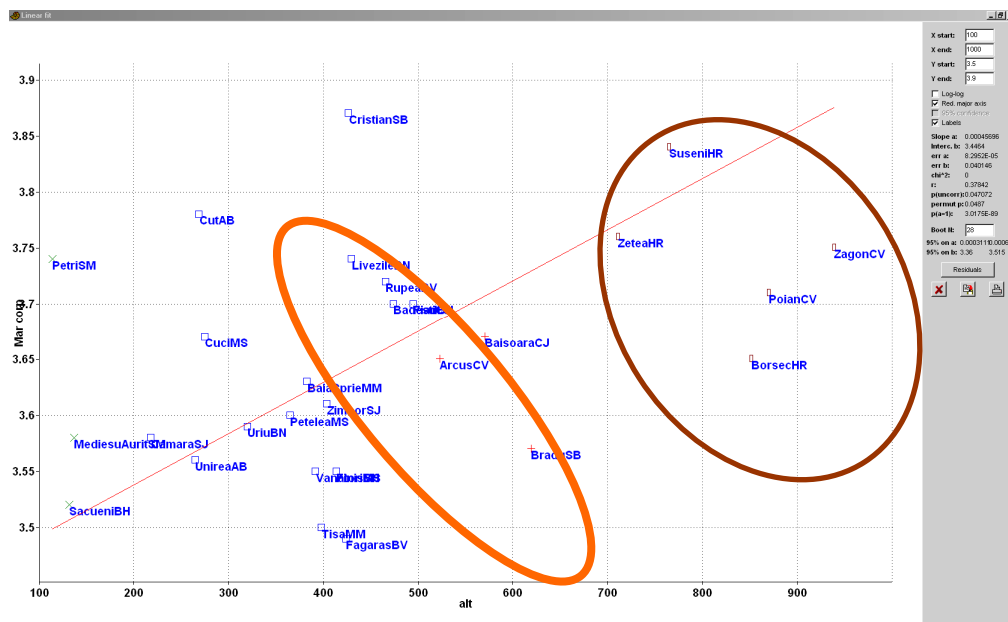


Figure 6 – Grouping of bees due to altitude (PAST ed. 1,85)

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 climacteric 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 3 has 3 activities:

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, Bradu SB, Zagoni CV. Only 13 samples out 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%), Petelea 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* subspecies, 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.

Table 3

Kruskal-Wallis test analysis results for the similarity between the samples

	CristianSB	LivezileBN	RupeaBV	BadestiCJ	FiadBN	ArcusCV	BaisoaraCJ	BradusB	ZeteaHR	SuseniHR	BorsechHR	PoianCV	ZagonCV
PetriSM	0.03932	0.36000	0.21480	0.62210	0.82060	0.96980	0.67760	0.93970	0.07412	0.41810	0.41810	0.18770	0.96980
SacueniBH	0.01145	0.75830	0.43030	1.00000	0.66820	0.57520	0.89510	0.50970	0.44040	0.85350	0.97550	0.97950	0.37340
MediesuAuritSM	0.00573	0.78430	0.78960	0.38110	0.14320	0.19770	0.31950	0.15140	0.81830	0.23920	0.41150	0.31210	0.10730
CamaraSJ	0.00873	0.97550	0.76480	0.51810	0.86910	0.69240	0.59780	0.64440	0.30360	0.92640	0.90200	0.60700	0.44830
UnireaAB	0.03442	0.54950	0.26430	0.77820	0.76240	0.96980	0.85010	0.96980	0.13540	0.54950	0.50350	0.36410	0.85010
CutAB	0.01380	0.86960	0.50520	0.89550	0.88800	0.72480	0.83270	0.69850	0.33800	0.97380	0.86960	0.78430	0.45970
CuciMS	0.00397	0.77530	0.83580	0.39180	0.12720	0.19240	0.33170	0.12720	0.77670	0.19450	0.39180	0.23010	0.08551
UriuBN	0.03071	0.69360	0.66390	0.94760	0.30720	0.45970	0.83270	0.36000	0.78430	0.49050	0.84380	0.60300	0.32420
PeteleaMS	0.01243	1.00000	0.73890	0.79430	0.40250	0.32110	0.64180	0.36850	0.78960	0.52390	0.75000	0.61040	0.20360
BaiaSprieMM	0.01693	0.85350	0.56790	0.92640	0.66820	0.53100	0.84320	0.48870	0.57150	0.80550	0.97550	0.85710	0.37340
VanatoriMS	0.07861	0.69360	0.68510	0.97380	0.80530	0.77820	0.97190	0.69850	0.52900	0.97380	0.97380	0.86950	0.64720
TisaMM	0.00655	0.95380	0.66290	0.75000	0.51490	0.47570	0.70980	0.35220	0.61040	0.70650	0.86200	0.75240	0.29170
ZimborSJ	0.03442	0.39810	0.17240	0.67270	0.70550	0.87980	0.70550	0.87980	0.07898	0.41810	0.36000	0.25350	1.00000
FinisBH	0.02080	0.74270	0.40090	1.00000	0.83270	0.94390	1.00000	0.86030	0.19830	0.66950	0.66950	0.49380	0.72480
FagarasBV	0.00573	0.95090	0.80660	0.51810	0.55290	0.62090	0.53100	0.37340	0.60700	0.75830	0.87770	0.81700	0.32260
CristianSB	0.00000	0.02703	0.01243	0.02703	0.03442	0.11580	0.05089	0.06520	0.00299	0.01586	0.02374	0.00261	0.06520
LivezileBN	1.00000	0.00000	0.97690	0.62240	0.54950	0.59740	0.59740	0.43860	0.70160	0.76760	0.76760	0.97820	0.43860
RupeaBV	1.00000	1.00000	0.00000	0.52390	0.21480	0.35220	0.47570	0.26430	0.94200	0.36920	0.66390	0.54410	0.21480
BadestiCJ	1.00000	1.00000	1.00000	0.00000	0.67270	0.64720	0.94390	0.64720	0.32440	0.97380	0.89550	0.66140	0.57320
FiadBN	1.00000	1.00000	1.00000	1.00000	0.00000	0.70550	0.82060	0.87980	0.25350	0.86030	0.62210	0.74740	0.57080
ArcusCV	1.00000	1.00000	1.00000	1.00000	1.00000	0.00000	0.82060	0.96980	0.30550	0.75130	0.69850	0.59820	0.82060
BaisoaraCJ	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.00000	0.73370	0.31950	0.94390	0.83270	0.68190	0.67760
BradusB	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.00000	0.19770	0.62210	0.54950	0.48230	0.87980
ZeteaHR	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.00000	0.35200	0.56540	0.37030	0.12070
SuseniHR	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.00000	0.84380	0.97820	0.48130
BorsechHR	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.00000	0.95630	0.45970
PoianCV	0.98600	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.00000	0.30550
ZagonCV	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	0.00000

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

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 (Figure 7).

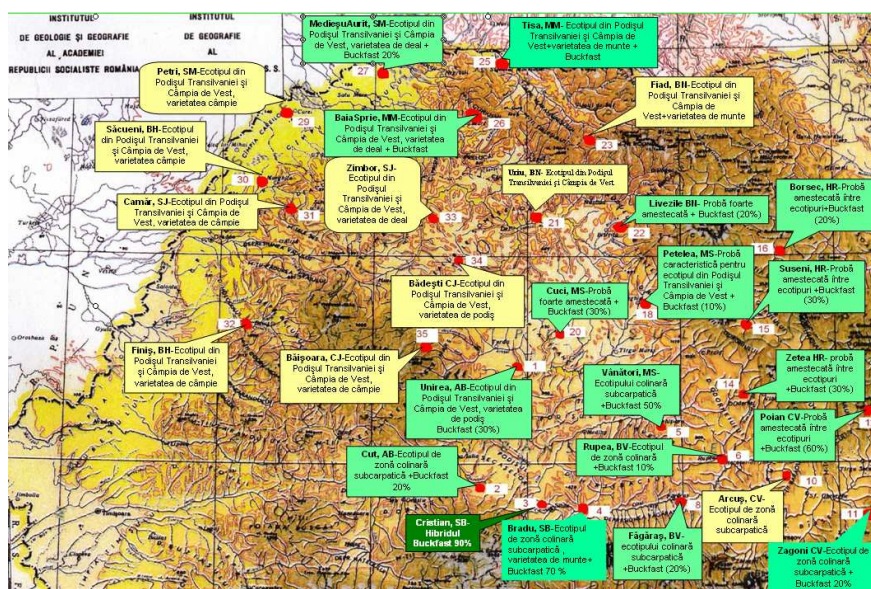


Figure 7 – Distribution of analyzed Transylvanian honeybees genotypes and varieties due to various microsatellite loci

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