University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca,
Faculty of Animal Husbandry and Biotechnologies,
3-5 Mănăştur Street, 400372, Cluj-Napoca, România

Characterization of genetic potential of the honeybee *Apis mellifera carpatica* using molecular techniques in order to conserve its biodiversity

Synthesis of Second Report

Director: Prof. Liviu Al. Mărghitaş, *PhD*

2008

The project entitled "Characterization of the genetic potential of honeybee *Apis mellifera carpatica* using molecular techniques in order to conserve its biodiversity"; for the period of the stage December 15, 2007 to October 31, 2008, our team has set the following objectives: 1. Determination of morphometric measurements in *Apis mellifera carpatica*; 2. Identification of molecular markers used in the testing methods of analysis of genetic variability of bees; 3. Testing of molecular markers and the choice of method of work.

To achieve these objectives were carried out following activities: 1. Purchase equipment and materials for measurement and data interpretation software needed; 2. Morphometric measurements are performed in *Apis mellifera carpatica* by computerized three biometrics; 3. Interpretation of data obtained in connection with specific references to race and international; 4. Biometric comparative study of five local ecotypes; 5. Identifying specific markers used in molecular analysis; 6. Purchase materials for molecular analysis; 7. Establishment of gene locuses on the gene which can provide accurate analysis of racial differentiation; 8. Practical applications and identification of the best work; 9. Analytical testing by molecular markers; 10. Variability analysis of microsatellite.

1. DETERMINATION OF MORPHOMETRIC MEASUREMENTS IN *APIS MELLIFERA CARPATICA*

To determine the morphometric measurements were used:

Biological material. Samples of bees were collected from different localities in Transylvania, from traditional beekeepers with apiaries, avoiding possible areas where pastoral practice beekeeping. It was very important because so tried to avoid mixing of genes between different populations in Romania. When collecting samples of biological material (honeybees) have considered the following factors: ► sampling period to obtain young bees; ► flying range of queens and drones for mating; ► harvesting a batch is done only within a hive, the combs; ► predominant color of the honeycomb to harvest. The collection was made from three different locations in each county. Only in Sibiu this collection was not possible because most beekeepers operate Buckfast hybrid or pastoral practice beekeeping. In other counties tried encompassing a significant area for each county and selected groups were positioned at a minimum distance of 50 km. The samples were collected by bees from the following locations: Unirea, Cut, Cristian, Bradu, Vânători, Rupea, Făgăraş, Arcuş, Zagoni, Poian, Zetea, Suseni, Borsec, Petelea, Cuci, Uriu, Livezile, Fiad, Tisa, Baia Sprie, Medieşu Aurit, Petri, Săcueni, Camăr, Finiş, Zimbor, Bădeşti şi Băişoara.

Chemicals (used for morphometric determinations and DNA extraction): Chelex 100 Sodium form. (Sigma), proteinase K (Promega) 2x PCR Master Mix (Fermentas or Promega); forward primer: B 124, 7, A 24, A 28, A 35, A 107, A 113, A 88 (Eurogentec); reverse primer: B 124, 7, A 24, A 28, A 35, A 107, A 113, A 88 (Eurogentec) agarose powder (Promega, or Merck), 10 X TBE buffer (Lonza), $MgCl_2$ (25 mM) (Promega), dNTP (10mm) (Promega), diethyl ether 74, 12 M g/mol (Merck); GoTaq polymerase 5 U/ml (Promega) ultra H_2O (Fermentas or Promega), color: 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 × (Invitrogen).

<u>Electronic devices and instrumentation</u> (used for morphometric determination and DNA extraction) and dissection kit Olympus SZX 16 stereomicroscope, Olympus Camera: Olympus SP-350, light source: KL 2500 LCD OLYMPUS, software Quick MICRO PHOTO 2.2, Analytical balance: PARTNER XA 110; small Centrifuge: Sigma 1-13; high Centrifuge: Hettich MIKRO 22-R; shaker Electric Biosan Orbitalshaker OS-10, Vortex: Heidolph REAX top; microwave device: Vortex; fridge: Arctic; PCR: Corbett RESEARCH and Eppendorf Mastercycler personal, electrophoresis line - current line EV 261 Consort, cell migration: Consort nv and transiluminator: UVP, Spectrophotometer: Beckman Coulter DU 730.

<u>Results:</u> The morphometric measurements were performed according to methods described by Ruttner (1988), but there were made some changes: remove bees from the freezer and dried with hot air water steam, the hair on them to break. Turn the light source and adjust the light intensity required to illuminate the microscope weight. Use stereomicroscope Olympus camera, which is connected to a computer. Fix the mass bee parts in microscope, adjust the magnitude and clarity of picture and then catch the camera. Magnitude must always have the same values, otherwise morphometric measurements will have incorrect values. Pictures appear on the computer monitor so the can be seen in *Figure 1*. Measurements taken at the head are: internal line simple eyes (POL), the line between compound eyes and simple eyes (OOL), lateral line of the eye (LOL), size labrum, trunk length. On the wing were determined following measurements: the 11 angles formed by combining rib wing: A4, B4, D7, E9, G18, i10, I16, K19, L13, N23, O26, previous wing length (FL), previous wing width (FB), the length of radial cell (RC) index cubital cubital vein composed of A (CV A) and cubital vein B (VC B). Rear wing (Figure 27) is measured as follows: hind wing length (APL), width rear wing (Laap), number of harness (H).

On the back foot is measured as follows: femur length (EF), tibia length (TI), metatarsal length (ML) width metatarsal (MT). To the abdomen were determined: length sternitului 6 (S 6), width sternitului 6 (LS 6), length sternitului 5 (S 5), length sternitului 4 (S 4). Also we process some measurements at tergite level.

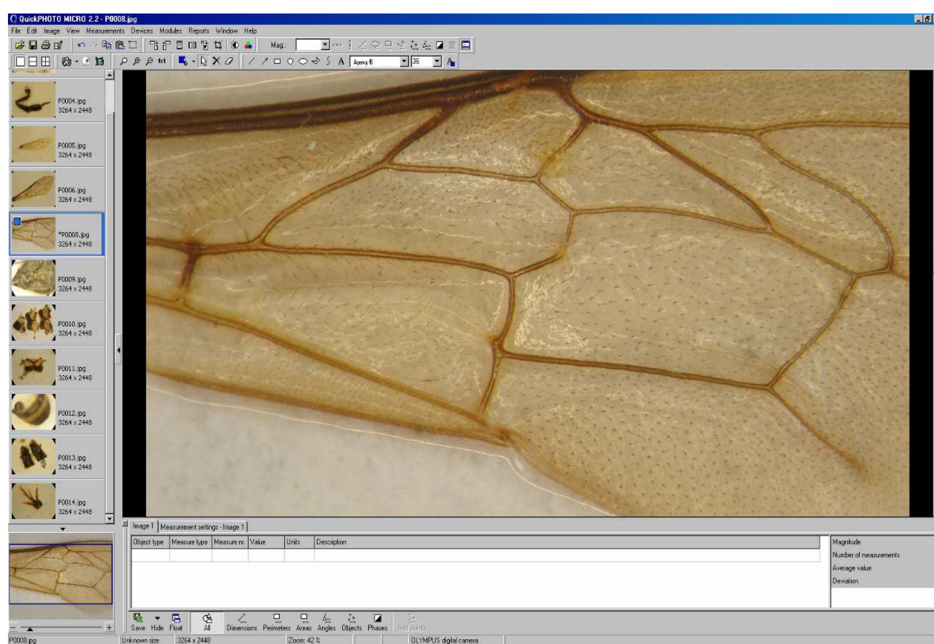

Figure 1. General Presentation of Quick PHOTO MICRO 2,2. program

The following figures are the number of measurements:

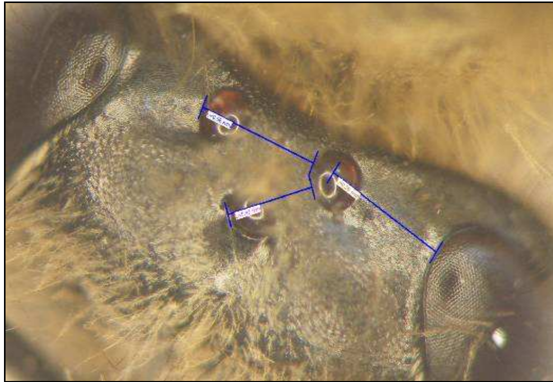

Figure 2. Simple and composed eyes (*ocelii*)

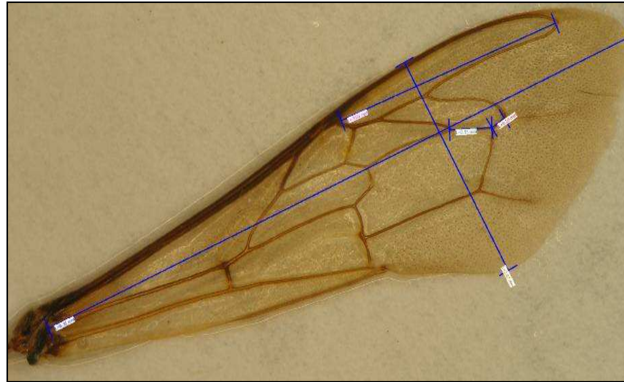

Figure 3. Firts wing measuremets

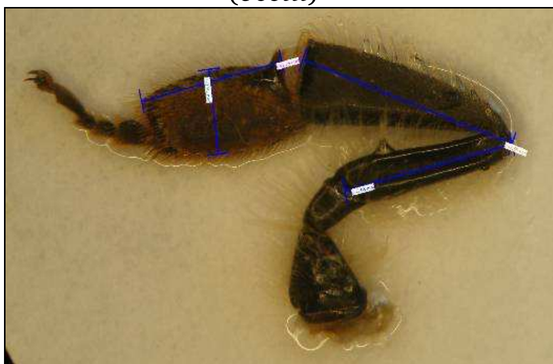

Figure 4. Posterior leg measurements

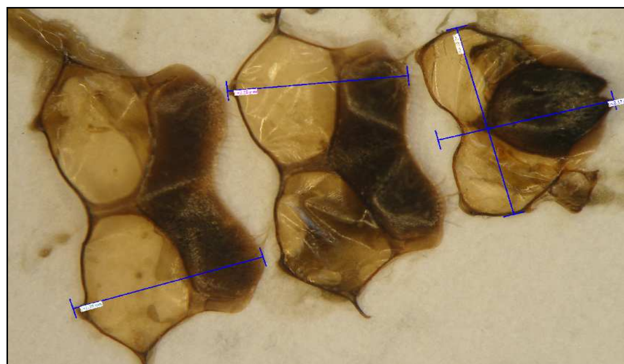

Figure 5. Sternit 4, 5 and 6 measurements

The morphometric measurements obtained by MICRO PHOTO Quick is saved in Excel 2.2 and statistical processing follows.

Following morphometric measurements and genetic analysis were obtained significant results by performing statistical analysis of these data. The literature distinguishes five ecotypes the bee *Apis mellifera carpatica* according to morphometric measurements, based on the following characters: length of trunk, length of body, length of tarsus and tibia, wing length earlier, cubital index. Bee ecotypes in our country are:

- Plain southern ecotype;
- plateau and plain ecotype of Western (including Banat)
- Carpathian ecotype of hilly;
- Plateau ecotype of Moldova;
- ecotype of Dobrogea Plateau (including Delta).

The analysis is taken within ecotype of the plateau and the Western Plain and Carpathian ecotype hilly area, in addition to these five ecotypes bees in Romania can be classified in different varieties: plain, hill and mountain. This adaptation is likely, considering the wide variety of environmental factors in this part of Transylvania, and if we take into account the laws of Bergmann, Allen and Glover on various animal species adapt to environmental conditions.

The objective of this study was to determine whether these ecotypes or varieties exists in Transylvania. Statistical analysis was performed using 1.84 EASTER program. Exe (Palaeontological Statistics, version 1.84 exe.) (Http://folk.unio .no / ohammer / paste) and the software Origin.

Thus could establish a correlation between environmental factors, as shown in Figure 6.

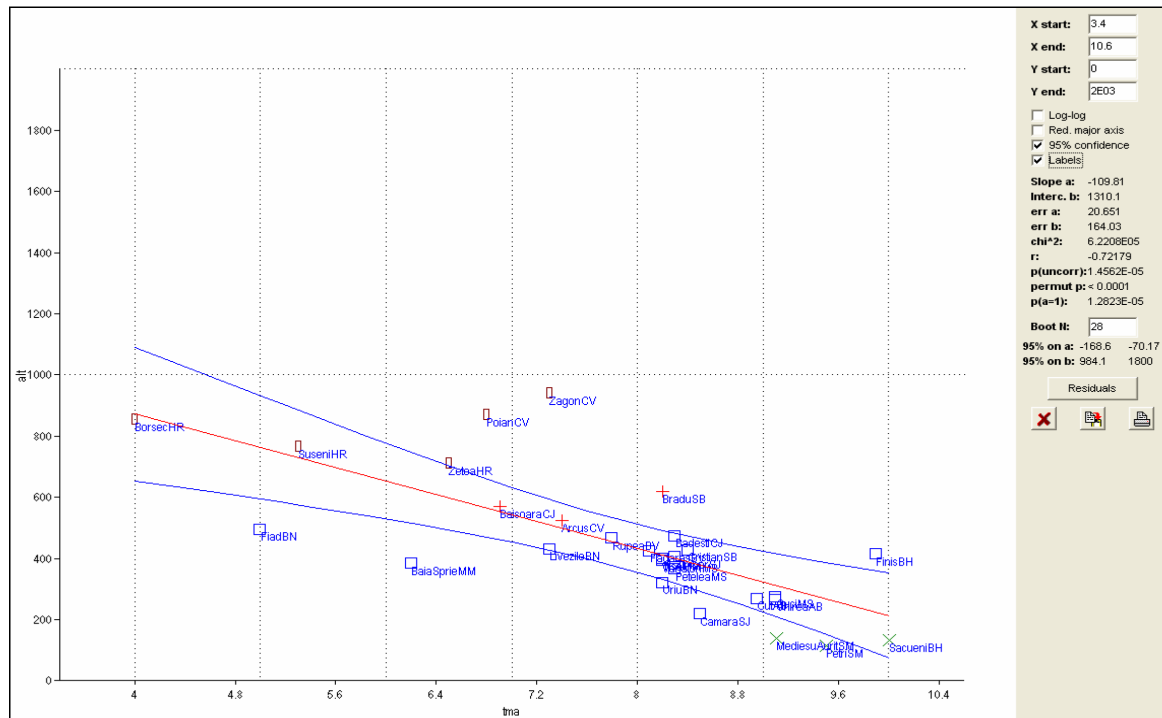

Figure 6. Linear correlation between environmental factors: altitude (altitude) and multi-annual average temperature (TMA). Linear regression equation is alt = -109.81, tma + 1310.1

For more accurate expression data using two-step procedure of multivariate analysis:

1. factor analysis (specifically principal components analysis - PCA - used to detect sub-groups in a population in a given area).

2. discriminatory analysis stage (YES - to confirm and identify subgroups established character of the most discriminatory in order to calculate the distance between center groups).

Tried to correlate data from morphometric measurements correlate with each other or with environmental factors. Morphometric measurements in the strings can get variations, but gives no specification for nature intended, instead gives us valuable data when they are linked to environmental factors. Correlation between environmental factors and nature should therefore be give us accurate data. So we wanted to correlate morphometric measurements with the laws of Allen and Bergmann Rensch. These laws include the animal kingdom, with particular application to bee. Allen's Law states that the lower legs and trunk length in cold climates. Rensch argues that annexes involved in protection temperature increase with altitude and latitude, so the animals in cold climates should have hair longer and more dense. Bergmann argues that the thorax increases with latitude and altitude.

After processing the data obtained it can be concluded that there is no correlation between characters measured and environmental factors. Allen Law, Rensch Bergmann and general concerns regarding the animal kingdom, but the same results were obtained by Ruttner when analyzing strains of bees worldwide. But these correlations cannot be observed from measurements made on samples of bees from Transylvania. The results obtained from morphometric measurements determinations do not discriminate against ecotypes identified during `70. Furthermore, no varieties of plain, hill or mountain may not be highlighted. This is explained by mixing ecotypes detected in more than 30 years ago due to intense beekeeping pastoral practice. Morphometric measurement method has been increased by genetic analysis that provides a more accurate classification at microsatellite level.

2. IDENTIFICATION OF MOLECULAR MARKERS USED TO TEST METHODS OF ANALYSIS OF GENETIC VARIABILITY OF HONEYBEES

To achieve this objective there were tested following molecular markers:

Sequence	Primer
5'-GGCAGAATAAGTGCATTG-3'	E 2
5'-CAATATCATTGATGACC-3'	H 2
5'-TCCACAAATAAAACCCCAAGATT-3'	N 1
5'-GAAGTGTAAGTGCAAAATATCTATG-3'	N 2
5'-CAACATCGAGGTCGCAAACATC-3'	16 s rDNA (ADNmt)_F
3'-AGTTGGGACTATGTTTTCCATG-5'	16 s rDNA (ADNmt)_R
5'-GATTACTTCCTCCCTCATTA-3	CO I_F
3'-AATAAGTCTGATAGGTCTAA-5'	CO I_R

These molecular markers have been described in many studies of foreign literature, and they are used widely in the world. Materials used in this experiment are presented in the first part of this exposure, using the classical methods of PCR determining for phylogenetic analysis using molecular markers. We tried first Phenol-chloroform method, but this is a very laborious and using toxic substances to human body. It was thus decided to use the Chelex method. This working method (Chelex method) identified as molecular markers for honey bees from Transylvania primers E2 and H2 (Garnery *et al.*, 1992). They will be used for following genetic analysis of different bee populations in Transylvania.

3. MOLECULAR MARKERS: TESTING AND SELECTION OF METHOD OF WORK

Were used more steps (analysis method) for testing of genetic variability in Apis mellifera carpatica from Transylvania using mitochondrial DNA, ie genomic DNA extraction, quantification of samples, PCR with specific primers, their migration agarose gel, image capture and analysis and final interpretation of data obtained for finding the degree of phylogenetic relatedness between populations analyzed.

DNA extraction. (Chelex method) was achieved by exploratory a relatively simple protocol, using the following reagents: Chelex, proteinase K, ethanol 70%, distilled water. As part anatomy for extraction were used thoracic muscles. After

working follow steps put in termocycler sample site with the following PCR program: 55℃ - 1 hour, 99℃ - 15 min., 37℃ - 1 min., 99℃ - 15 min. Then samples were stored in refrigerator at 4℃ to amplify the desire d fragment of interest.

DNA quantity and purity has read using NanoDrop 1000 spectrophotometer. The results show little difference in status between different purity samples analyzed (Table 1). Quantity and purity of DNA amplification can be considered good for them and analysis of mitochondrial DNA sequences.

Was based on **PCR** using several sets of primers "universal" (which were already tested in several European research teams, and have yielded good results for biodiversity analysis in populations of *Apis mellifera* breeds: *Apis mellifera lingustica*, *Apis mellifera caucasica*, *Apis mellifera carnica*. Image capture was performed from agarose gel (where they migrated mtDNA bands) (Fig. 7) using the UVP system proceed to estimate the number of base pairs according to molecular size marker used (ladder, 100bp, Promega, USA).

Table 1

Quantity (ng / ml) and purity (260/280) of DNA extracted from thoracic muscles of Apis mellifera Carpathian breeded in different counties in 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

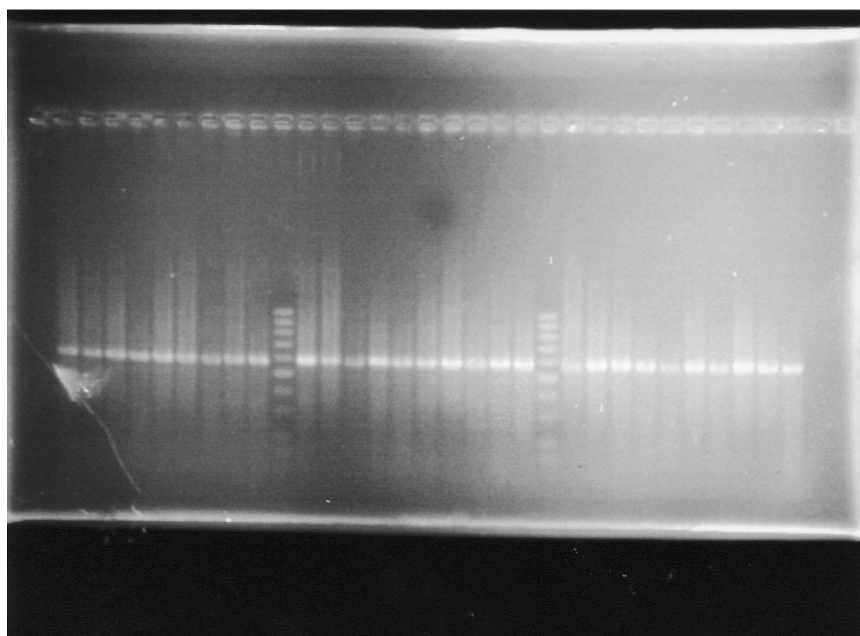

Figure 7. mtDNA bands migrated on agarose gel

Samples migrated to test this protocol came from Cluj County, Băişoara, Bădeşti and Deuş localities. The method has been successfully tested, the protocol is appropriate and in subsequent stages of the project will be applied for the remaining samples for phylogenetic characterization of honeybee population in Transylvania.

Selective Bibliography

DuPraw E. J., (1965a) Non-Linnean taxonomy and the systematics of honeybees, Syst. Yool. 14, 1-24.

DuPraw E.J. (1965b) The recognition and handling of honeybee specimens in non-Linnean taxonomy, J. Apic. Res. 4, 71-84.

Fisteag J.N. (1937) Cercetări Biometrice la Albinele Româneşti, Diss. Nr. 606. d. vet. med. Fac. d. Universitiit Bukarest..

Garnery L., Cornuet J.M. and Solignac M. (1992) Evolutionary history of the honey bee *Apis mellifera* inferred from mitochondrial DNA analysis. Mol. Ecol. 1, 145–154.

Kandemir I., Kence M., Kence A. (2000) Genetic and morphometric variation in honeybee (*Apis mellifera* L.) populations of Turkey. Apidologie 31, 343–356.

Liviu Alexandru Mărghitaş (2005) Albinele şi produsele lor, Editura Ceres, Bucureşti.

Moritz R.F.A., Cornuet J.M., Kryger P., Garnery L. and Hepburn H.R. (1994) Mitochondrial DNA variability in South African honeybees (*Apis mellifera* L.). Apidologie 28, 169–178.

Rinderer E. Th., ed. (1986) Bee Genetics and Breeding. Academic Press Inc.

Rortais A, Analzse de la biodiversité de l'abeill en France, Abeilles and Flures 2006, 24-27.

Ruttner F. (1988) Biogeography and Taxonomy of Honeybees, Springer-Verlang, Berlin.

Ruttner F., Tassencourt L., Louveaux J. (1978) Biometrical-statistical analysis of the georaphic variability of *Apis mellifera L.*, Apidologie 9.

www.eurohonig.cam