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PRELIMINARY RESEARCHES REGARDING THE GENETIC AND MORPHOMETRIC CHARACTERIZATION OF HONEYBEES (*A. MELLIFERA L.*) FROM ROMANIA

CERCETARI PRELIMINARE PRIVIND CARACTERIZAREA GENETICA SI MORFOMETRICA A ALBINELOR (*A . MELLIFERA L.*) DIN ROMANIA

CAUIA ELIZA*, USURELU DANIELA**, MAGDALENA LAURA MONICA**, CIMPONERIU D.**, APOSTOL POMPILIA**, SICEANU A. *, HOLBAN ALINA**, GAVRILA L.**

** Institute of Beekeeping Research and Development, Bucharest, România*
*** Institute of Genetics – University of Bucharest, România*

The international investigations regarding the honeybees' diversity carried out until now have revealed a certain degree of genetic pollution in different countries from Europe, because of the import of more productive honeybees' races or of some interracial honeybees' hybrids. This fact might have a negative impact on the success adaptability of honeybees at the ecosystem. Although, the Romanian honeybees (Apis mellifera carpathica) are well adapted to the local conditions and express a good resistance to diseases, the introgression (genetic pollution) of different honeybees' races could be an imminent event. So that, starting from 2007, by a cooperation between the Institute for Beekeeping Research and Development from Bucharest and the Institute of Genetics of the University of Bucharest, we have initiated different investigations in order to obtain a more accurate state of the Romanian honeybees' diversity. We have performed specific molecular analyses, using mtDNA (the COI-COII test) extracted from 32 different honeybees samples collected from several regions from Romania. For a better and detailed characterization of the collected honeybee's samples we have also carried out some morphometric measurements of their wings. Our data have shown that the Romanian population of honeybees is almost homogenous from the genetic and the morphometric points of views. These types of investigations represent a premiere for Romania.

Key words: honeybee, *Apis mellifera carpathica*, mtDNA, morphometry, population characterization, ecotype.

Introduction

For a long period of time, the morphometric techniques have contributed in a large measure to the discrimination between different honeybees' subspecies in order to characterise their diversity (Ruttner F., 1988). Despite the fact that these techniques are very laborious, they are still used. One reason for this is that the morphometric techniques have been permanently improved because new computational and imagistic techniques and even sophisticated software appeared. In parallel with the genetic and the molecular biology progresses from the last decades, new investigation tools such us genetic markers were developed. The genetic markers are very informative and relevant for an accurate characterization of the honeybees' population. Nowadays, the genetic markers are largely used for studying the biogeography of *A. mellifera* races and for establishing the degree of introgression of other non local races in: France – by Garnery L., Germany – by Moritz R., Spain -by de la Rua P., Brasilia – by Collet T., Slovenia – by Susnik S., Greece – by Harizianis P. In this context, we started similar studies by a research project, funded by MEdC (Module 2 CEEX, 2006-2008), between the Institute of Beekeeping Research and Development and the Institute of Genetics - University of Bucharest. The aim of our studies is to optimise and to implement the new techniques in order to characterise the Romanian honeybees' population.

A series of studies, performed until now, regarding the honeybees populations characterisation were based on the established informative molecular markers from two levels: the level of the mitochondrial DNA (mtDNA) which includes the CO I-CO II region and the nuclear level, by analysing the microsatellites. The COI-COII intergenic region contains an important length polymorphism of basic sequences, which discriminate the lineages of *A. mellifera* by the combinations of the 3 related sequences: *Po* (67/68 bp), *P* (54 bp), *Q* (192-196 bp), which are: *PoQ*, *PoQQ*, *PoQQQ*, *PQ*, *PQQ*, *PQQQ* and *Q*. The three mtDNA lineages are characterized by Po in A lineage, P in M lineage and neither by Po or P, only Q in C lineage (fig. 1). The length variability of the COI-COII intergenic region is combined with a restriction polymorphism site, which favored the application of a rapid and simple test for characterization of the mtDNA haplotypes. This test includes a PCR step of the COI-COII region followed by a *DraI* restriction of the amplified fragment. (Garnery et al, 1993).

Some studies done by Ruttner F. (1988), based on morphometry, show that honey bees from Romania belong to *A. m. carnica* subspecies and *A.m. macedonica* in the southern part of the country *(C lineage),* but other studies (Fotii N. 1965) show that there are some differences as compared to *A. m. carnica* and named it *A. m. carpathica*. Taking these into account, our goal was to establish the phylogenetic affiliation of the Romanian honeybees and the identification of some differences (haplotypes) by genetic markers in correlation with some morphometrical measurements. Our preliminary investigations have successfully implemented the CO I - CO II test in parallel with some morphometrical measurements on honeybees collected from different regions of Romania.

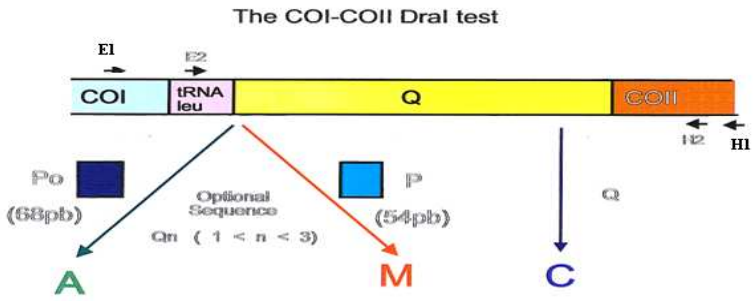

Figure 1. The structure of the COI-COII intergenic region
(According to Garney L., personal communication)

Materials and Methods

A number of 32 honeybees samples were collected from different apiaries located in the following Romanian counties: Brasov, Dolj, Bistrita, Gorj, Iasi, Prahova, Hunedoara and Constanta (fig. 2). From each county there were collected 4 honeybee samples, using a specific protocol for collecting and preserving the honeybees, according to the literature (Garnery, 1993).

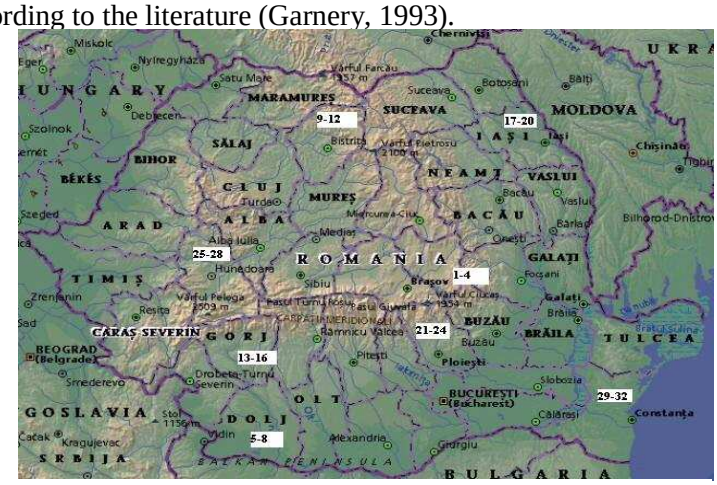

Fig. 2 The distribution of analysed honeybees samples around Romania

The implementation of the COI-COII test

The DNA was extracted from the heads of the collected honeybees using the Wizard Genomic DNA purification kit for animal tissue (Promega). The amplification of CO I - CO II region by PCR was done using two sets of primers: the first set of primers is: **E1**:5'GATCAATATCATTGATGACC3' / **H1**: 5'TCTATACCACGACGTTATTC3' (Hall and Smith, 1991) and the second set of primers is: **E2:** 5'GGCAGAATAAGTGCATTG3' / **H2**: 5'CAATATCATTGATGACC3'. (Garnery, 1993).

The two sets of primers were necessary to cover different borders of the COI-COII region, which increases the possibility to found polymorphisms, revealing different Romanian honeybee's ecotypes.
The PCR fragments were checked in a 2% agarose gel electrophoresis in order to determine the size of the amplified fragments.
The amplified fragment was digested with DraI restriction enzyme, for 12 hours at 37^{o}C. Restricted DNA fragments were separated using a 10% acrylamide gel, stained with ethidium bromide.
The same honeybees samples, analyzed previously at the mtDNA level, were prepared for some morphometrical analyses made on the right fore wing. The fore wings of the honeybees' samples were mounted on the microscopic slides and their images were taken out and processed with the help of a stereomicroscope (Motic) that includes a digital video camera with a specific software (Motic Images Plus 2.0). The measurements were done after the calibration of the measurement function included in the camera software. The following measurements were performed: the L1 and L2 of the cubital cell, and the L3, L4 of the radial cell according to the literature [14]. Following these measurements, the cubital and Hantel indexes were calculated and discoidal shift was established, these indexes being widely used for honeybees' population characterisation.

Results and Discussions

The COI-COII test. By using *the first set of primers,* the PCR reaction revealed amplification fragments with the same molecular size (800bp) for all the samples (fig. 3). Moreover, the Dra I restriction, checked in 10% PAGE, showed that the DNA restriction fragments have the same molecular size (fig 4). In this way, by using the first set of primers, no differences between the samples were identified and we couldn't observe different honeybee ecotypes on selected Romanian areas.

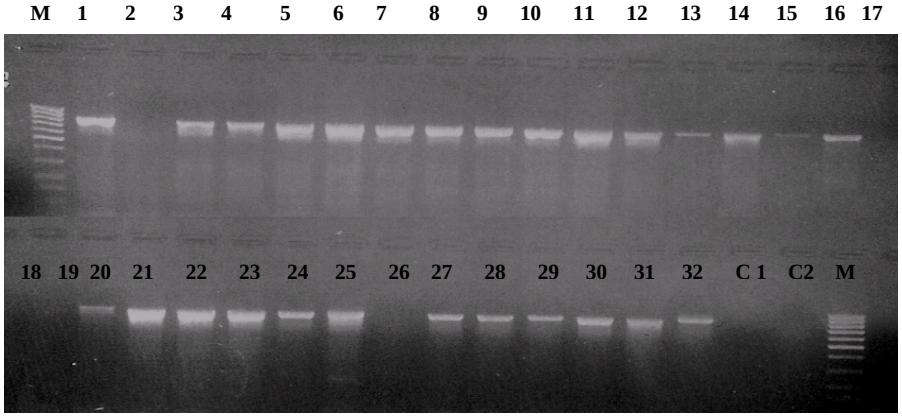

Fig. 3 Electrophoregrame of PCR amplified products (the E1H1 primers)
Lines 1-32 - E1H1amplicons (800 bp); M - molecular marker 100-1000pb,
C1= negative control (without DNA), C2 = control for DNA specificity (using human DNA); (agarose gel electrophoresis 2%, TBE 1x)

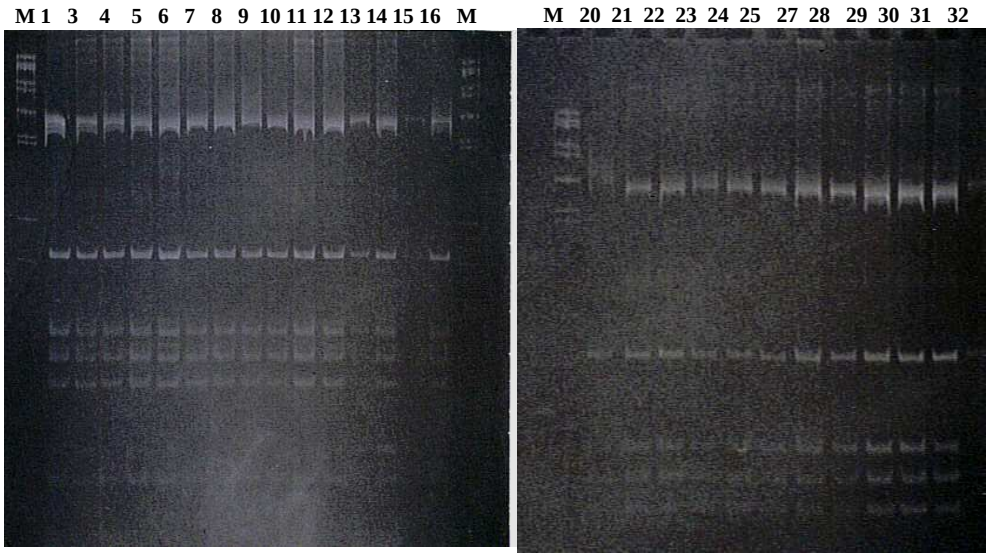

Fig. 4 Electrophoregrame of RFLP fragments obtained after restriction with DraI
Lines 1-32 – restriction pattern obtained from E1H1 amplicons;
M - Molecular marker 100-100pb; (10% acrylamide gel, TBE 1x)

The obtained results using *the 2nd set of primers* are shown in the figs.5&6. Using this set of primers we obtained again PCR products having similar size and similar pattern of migration for the collected samples (fig 5).

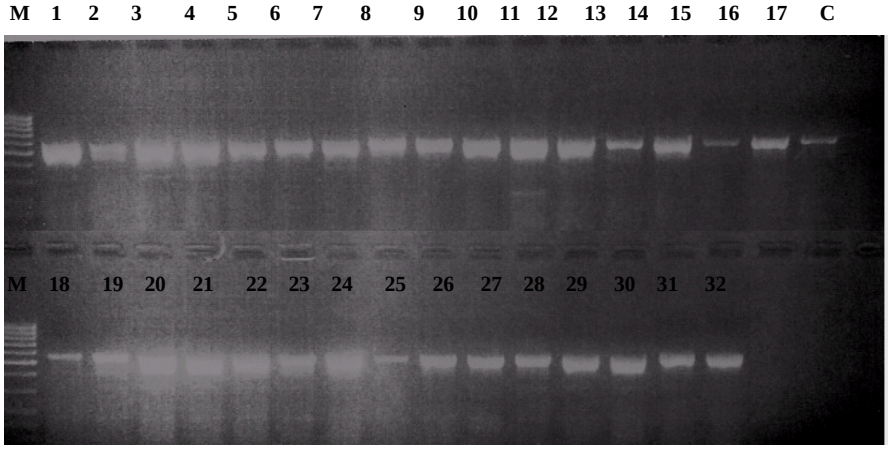

Fig. 5 Electrophoregrame of PCR products using the second set of primers
Lines 1-32 - E2H2 amplicons (536 bp); M - molecular marker 100-1000pb,
C - Control; (agarose gel electrophoresis 2%, TBE 1x)

Performing the restriction reaction using Dra I enzyme on E2H2 amplified fragments we didn't notice a different electrophoresis pattern between the samples (fig. 6).

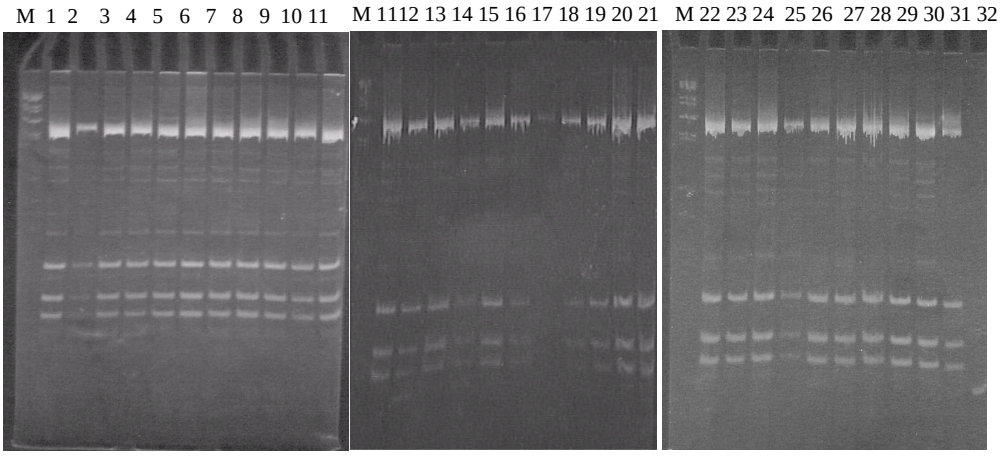

Fig. 6 RFLP fragments obtained after Dra I restriction on E2H2 amplicons
Lines 1-32 – Restriction fragments obtained for samples 1-32,
M - Molecular marker 100-100pb; (PAGE 10%, TBE 1x)

For the first set of primers we couldn't compare our data with those existing in the literature because almost all the works done in the field were done on M and A lineage of *A. mellifera*. In Romania this is the first molecular study performed on local honeybee. Based on our preliminary results regarding the molecular polymorphism in the COI-COII region (for the E1H1 amplicons) we determined that Romanian honeybees are still a homogeneous population.

Using the second pair of primers we noticed that the obtained amplified fragments have a molecular size (536 bp) comparable with the ones often reported by Garnery et al. (1993, 1998, 2000, 2001) for C lineage. In their studies there are investigated samples from France and other countries, neighboring to Romania (i.e. Bulgaria, Slovenia, Hungary), which have the C lineage of honeybees. In the same way, our results obtained after Dra I restriction on E2H2 amplicons could be compared with those determined by Garnery and collaborators (41 bp, 47 bp, and 65 bp) (Garnery et al., 1993). Thus, based on our preliminary results and on the literature data it is feasible to affirm that the Romanian honeybees belong to the C lineage of honeybees.

Despite the geographical and ecological variations of the collection sites (plain, mountain, hill and delta) the analyzed Romanian honeybees samples didn't express population variation differences at the mtDNA level (i.e. different ecotypes).

In order to have a complete image regarding the genetic characterization, some further researches for microsatellites markers are going to be carried out, which could reveal possible differences between samples at the nuclear level.

The morphometric measurements

The calculated indexes (table 1) obtained following the morphometric measurements were compared with those from the literature (table 2).

County	***Honeybees samples***	***Cubital index L2/L1***	***Hantel index L4/L3***	***Discoidal shift***
Brasov	*1*	*2.1*	*0.98*	+
	2	*2.04*	*0.95*	+
	3	*2.59*	*1.13*	+
	4	*2.62*	*1.03*	+
	Average	**2.3375**	**1.0225**	
Dolj	*5*	*2.31*	*0.98*	+
	6	*2.43*	*0.9*	+
	7	*3.23*	*0.82*	+
	8	*2.18*	*0.97*	+
	Average	**2.5375**	**0.9175**	
Bistrita	*9*	*2.71*	*0.98*	+
	10	*2.2*	*0.89*	+
	11	*2.36*	*0.86*	+
	12	*3.145*	*0.91*	+
	Average	**2.60375**	**0.91**	
Gorj	*13*	*2.72*	*0.93*	+
	14	*2.28*	*0.94*	+
	15	*2.96*	*0.96*	+
	16	*2.55*	*0.87*	+
	Average	**2.6275**	**0.925**	
Iasi	*17*	*2.63*	*0.95*	+
	18	*2.94*	*0.94*	+
	19	*2.63*	*0.84*	+
	20	*2.19*	*0.89*	+
	Average	**2.5975**	**0.905**	
Prahova	*21*	*2.98*	*1*	+
	22	*1.92*	*1.05*	+
	23	*2.71*	*1.04*	+
	24	*2.6*	*0.99*	+
	Average	**2.5525**	**1.02**	
Hunedoara	***25***	***2.42***	***0.88***	**+**
	26	*2.67*	*0.95*	+
	27	*2.79*	*0.95*	+
	28	*2.24*	*0.92*	+
	Average	**2.53**	**0.925**	
Constanta	*29*	*2.88*	*0.96*	+
	30	*2.89*	*1.03*	+
	31	*2.59*	*0.96*	+
	32	*2.19*	*0.94*	+
	Average	**2.6375**	**0.9725**	

Table no 1.The obtained data regarding the morphometrical indexes done on 32 samples of honeybees from 32 different apiaries from eight counties

Indexes/races	***A.m. carnica***	***A.m. mellifera***	***A.m. ligustica***	***A.m. caucasica***
Cubital index	2.7(2.4-3.0)	1.7 (1.3-2.1)	2.3 (2.0-2.7)	2.0(1.7-2.3)
Hantel index	0,925-1,200	0.600-0.923	Not found	Not found
Discoidal shift	+	-	+	0

Table no 2. The reference morphometrical indexes found in the beekeeping literature

Looking at the obtained cubital indexes, we noticed that the values varies between 1,92 and 3,145, the most values being ranged between 2,5 and 2,7. Comparing the

obtained data with the specific indexes of some European honeybee races we noticed that our data regarding the cubital indexes are mostly in the range of *A.m. carnica* values: 21 samples have greater values and 11 samples have lower values than 2.4 which is the minimum of the interval range specific for *A.m. carnica*. Regarding the Hantel index, the values are ranged between 0,82 and 1,13, the medium values being mostly between 0,91 and 1,02. We noticed that 9 samples out of all samples have lower values than the minimum value characteristic for *A.m.carnica,* which could be characteristic for *A.m. mellifera* or for other races, which we don't have the comparison indexes for. We noticed also that only in 3 samples the two indexes are correlated, r=0,27 (samples no 10, 11, 20). Regarding the discoidal shifts the results show that it is positive in all samples so it shows that the samples belong to phylogenetic line C, most probable to *A.m.carnica* race. As these indexes are partially informative it is very obvious that it is necessary to increase the number of measurements of the body parts, as are described in the literature, and to apply them on a greater number of honeybees samples and locations from Romania and on honeybees collected from the other races, too.

Conclusions

1. There were performed molecular and morphometric analyses on 32 different samples of honeybees collected from different locations and 8 counties.
2. Our preliminary molecular investigations of the polymorphism from the COI-COII mitochondrial DNA region (with bought sets of primers) showed that Romanian honeybees are still a homogeneous population.
3. The morphometric results expresses that the majority average values of cubital index are ranging between 2.5 and 2.7, being characteristic for *A.m. carnica*;
4. The Hantel index values are more varied and belong to *A.m.carnica* (23 samples) as well as to *A.m. mellifera* (9 samples from different counties) and show that this index is not correlated with cubital index (r=0,27), only 3 samples with low Hantel index (the samples no 10, 11, 20) being correlated with a low cubital index.
5. The discoidal shift was positive and confirms the interpretation that from the morphometrical point of view the most values are in the range of the phylogenetic lineage C of *A. mellifera*, this fact being confirmed by genetic mtDNA analyses, too.

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