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RESULTS ON THE IDENTIFICATION OF THE HONEYBEE SUBSPECIES FROM SOME SOUTH AND SOUTH-EAST ROMANIAN COUNTIES USING A SEMIAUTOMATIC SYSTEM FOR ANALYZING WINGS

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Abstract

The geometric morphometric analysis proved to be an efficient and rapid method for the identification of honeybee subspecies. This analysis involves several steps but takes less than classic morfometric methodes being easier to apply. The aim of this study was to identify the honeybee subspecies from the Romanian South and South-Eastern area, using the semiautomated french system ApiClass, system that allows to identify the subspecies based on the wing's image of Apis mellifera honeybee worker. This program is analyzing the wing considering the 19 points corresponding of the intersections of the main veins of the honeybee forewings. Using ApiClass we analized 13 samples from Tulcea, Calarasi, Giurgiu, Olt, Dambovita, Prahova and Ialomita counties. Each sample was composed from wings of 20 honeybees. Five of the analysed samples were identified as hybrids without being possible to specify the type or the level of the hybridization and 8 samples had been identified as being Apis mellifera carnica presenting a homology degree over 90%.

Key words: *honeybee wing, Apis mellifera, geometric morphometric analysis.*

INTRODUCTION

Apis mellifera honey bees are found throughout Europe, Africa and Western Asia (Alattal et al., 2014). *Apis mellifera* is one of the most studied invertebrates. This species is distributed all over the world and has been distributed throw humans to numerous countries (Arias et al., 2008). Honeybees are usually classified into about 30 subspecies (Leno da Silva et al., 2015) and are grouped into four phylogenetic lines: A, C, M and O. Every line is associated to a geographical area (Rinderer, 2008). The O line (the Middle East group) includes the *Apis mellifera: anatolica, adami, cypria, syriaca, caucasica, meda.* The line M (western and northern European Mediterranean group) includes the *Apis mellifera: mellifera, iberica* subspecies. The line C (Central and Southern European Group) includes the *Apis mellifera: sicula, ligustica, carnica (the Carniolian bee), macedonica, cecropia* and line A includes African breeds of *Apis mellifera* (Tropical Africa, North Africa): *scutellata*, *adansonii, litorea*, *monticola, lamarckii*, *capensis, unicolor*, *yemenitica, sahariensis*, *intermissa*, *major* (Ruttner, 1988; Rinderer, 2008). This sub-species are ussualy described as - geographic sub-species - because their distributions correspond to a distinct geographic areas. Commercial breeding and also migratory beekeeping have affected the genetic variability of local honey bee populations (Bouga et al., 2011).

The honeybees express an important geographical variation, resulting in adaptation to regionally factors of climate and vegetation being particularly sensitive to inbreeding (Meixner et al., 2013).

The honeybee subspecies. The honeybee subspecies could be classified using morphometric characters, being available a lot of studies on this issue. The main morphological featurescan be classified into three major groups: length measurements, color measurements, and wing vein characteristics. The morphology characteristics is used to characterize honey bee races and individuals, to determine the degree of hybridization with foreign races and/or for the discrimination between honey bee subspecies (Aboushara et al., 2013). Wings are considered the most

reliable morphological feature for honey bees identification, so lately, the measurements of the fore wings have proven to be reliable for the honeybees' classification (Leno da Silva et al., 2015; Koca and Kandemir, 2013). There were developed different methodes for automatic honeybee classification based on images of the wings. Also, the geometric morphometry demonstrated to be a useful tool in terms of time consuming and reliability of the results (Leno da Silva et al., 2015).

Different software packages were developed for automated identification of the honeybee subspecies. Some of this software are: ABIS (Automated Bee Identification System) (Leno da Silva et al., 2015); ID-BEES; FABIS (Nawrocka et al., 2017); DrawWing system (Tofilski, 2008); ApiClass system (Nawrocka et al., 2017; Baylac et al., 2008). DrawWing and ApiClass software are available to the general public (Nawrocka et al., 2017).

MATERIALS AND METHODS

The aim of this study was to identify the honeybee subspecies from some South and South-East Romanian counties area, using the geometric morphometric method. For this purpose it has been used the semiautomated french system ApiClass. This system allows to identify the honeybees subspecies based on wings' picture of the *Apis mellifera* honey bee worker and is using the recent advances of geometric morphometry. ApiClass, analyzes the wing comparing the 19 points corresponding of the intersections of the main veins of the bee forewings (http://apiclass.mnhn.fr).

The coordinates are processed before being analyzed by the system. After that, the Apiclass system returns the probability of belonging to one of the honeybee subspecies from it reference system. This system has more than 5000 honeybee wings of the honeybee main lines and subspecies. Using the ApiC
lass system we analized samples from Tulcea, Calarasi, Giurgiu, Olt, Dambovita, Prahova and Ialomita counties. Each sample was composed from wings of 20 honeybees.

We used a slide scanner OpticFilm 7400 Plustek, pipettes, forceps, glass blades and small glass blades, Petri dishes, distileted water, collector tubes and alchool 70°.

Every honeybee forewings was scanned and then transformed in to jpg image being analyzed with ApiClass system. We select the automatic measuring system, the wing image was inserted into the system, this positioning points A, B, C on the wing in three steps. After this steps, the system is processing the , is placing the 19 points on the wing, is validating and analyzing the imageand its comparing it with the database of the system.

The analyzed honeybees came from the following counties/localities: Tulcea (Jijila, Luncavita, Pecineaga), Calarasi (Oltenita and Cocon), Giurgiu (Gostinu), Dambovita (Gura Ocnitei and Pucioasa), Olt (Visina, Bals), Prahova (Sinaia, Breaza) and Ialomita (Marsilian).

RESULTS AND DISCUSSIONS

To consider that a honeybee belongs to a particular subspecies of *Apis mellifera* the homology must be over 90%. Eight of the analysed samples had been identified as being *Apis mellifera carnica* (homology degree over 90%). Five of the analysed samples were identified as hybrids without being possible to specify the type or the level of the hybridization. Analysing the wings with ApiClass system the following were observed:

- in Tulcea county, the samples from Jijila, Luncavita and Pecineaga were identified as hybrids without being possible to specify the type or the level of the hybridization (see Tables 1 to 3);

Table 1. Analysed wings sample from Jijila - Tulcea County

Wing	Line	Jijila - Tulcea							
		Carnica	Ligustica	Cecropia	Buckfast	Mellifera	Caucasica	Cypria	Anatolica
1	C	45	24						15,23
2	C	98,09	2				0,79		
3	C	92,11	1,4				44,68		
4	C	98,05	0,85				0,88		
5	C	91			7,8	0,8			
6	O		10,22		84				
7	C	53	12,76			10,11			
8	C	64			54,78				
9	C	79,45				8,44			
10	C	83	11				31,14		
11	C	34		29					
12	C	23	2,01			29,92	20,35		
13	C	84					31,55		4
14	C	46	18,02		10,34				
15	C	89					31		
16	C	69			22,33		10,12		
17	C	53				34			
18	C	79,98	15,01		11				
19	O	90	2,67						
20	O			15,1			71		

**Line O - *Apis mellifera* - the Middle East group: *anatolica*, *adami*, *cypria*, *syriaca*, *caucasica*, *meda*;

*Line C - *Apis mellifera* subspecies Central and Southern European Group: *sicula*, *ligustica*, *carnica* (the Carniolian bee), *macedonica*, *cecropia*.

Table 2. First analyzed wings sample from Luncavita - Tulcea County

Wing	Line	Luncavita - Tulcea							
		Carnica	Ligustica	Cecropia	Buckfast	Mellifera	Caucasica	Cypria	Anatolica
1	C	33,04	73				18,74		
2	C	95	0,64				1		
3	C	10	52						1,19
4	C	89,11	25				0,88		
5	C	34	76						
6	O		15		3,24				79
7	C	62	26			10,11			
8	C	36			61,2				
9	C	80			8,99				
10	C		59		36				
11	C	50			12,99		29,43		
12	C	33,78	11		29,92		20,35		
13	C	77			3,56		30		
14	C	48,98			11		20		
15	C	82			26				
16	C	68	29				11		
17	C	51,41	37						
18	C	81	19		14				
19	O	81,98	4,5						
20	O			17	72				

**Line O - *Apis mellifera* - the Middle East group: *anatolica, adami, cypria, syriaca, caucasica, meda*;
*Line C - *Apis mellifera* - Central and Southern European Group: *sicula, ligustica, carnica* (the Carniolian bee), *macedonica, cecropia*.

Table 3. Second analyzed wings sample from Pecineaga - Tulcea county

Wing	Line	Pecineaga-Tulcea							
		Carnica	Ligustica	Cecropia	Buckfast	Mellifera	Caucasica	Cypria	Anatolica
1	M	42				20,01	22		
2	C		12,45			31,65		13,91	13,46
3	C	76,12		23,03					
4	O	34,28					70,11		
5	C	49,76					47		1,89
6	C	60		10,54		15,2			11
7	O	34,11					50		10
8	C	48		11,6			42		
9	C	98					0,27		
10	O	97,54			1,21		43		
11	M	19,65				41			
12	C	93,14	2,43						0,5
13	C	66			8,77		11		
14	C	93,41	10				4,55		
15	C	49,65			16,98		5,98		
16	C	92			0,31		0,56		
17	O	25			6,61		66,31		
18	C	90			10.00				
19	C	96	13,54		9,1				
20	C	91				5,43			

**Line O - *Apis mellifera* - the Middle East group: *anatolica, adami, cypria, syriaca, caucasica, meda*;
*Line C - *Apis mellifera* - Central and Southern European Group: *sicula, ligustica, carnica* (the Carniolian bee), *macedonica, cecropia*.
***Line M - *Apis mellifera* - Western and Northern European Mediterranean group: *mellifera, iberica* subspecies.

- in Calarasi county, the samples from Cocon (17 wings from 20) presented a homology degree over 90% with *Apis mellifera carnica,* instead the samples from Oltenita were hybrids (see Tables 4 and 5);

Table 4. Third analyzed wings sample from Oltenita - Calarasi County

Wing	Line	Oltenita - Calarasi							
		Carnica	Ligustica	Cecropia	Buckfast	Mellifera	Caucasica	Cypria	Anatolica
1	C	89			4				6
2	O	14							88
3	C	77					19		7
4	C	89				7			2
5	C	60		2			21		
6	O	11							79
7	M					82	4		10
8	M					69		14	
9	C	35				18	15		
10	C	56				20			
11	C	91			14				1
12	M					79			19
13	C	49					22		11
14	O	14							76
15	C	85			23			5	
16	M	17		5		69			
17	C	71			3		17		
18	O	14							84
19	C	91	12						
20	O	10							77

**Line O - *Apis mellifera* subspecies the Middle East group includes the *Apis mellifera*: *anatolica, adami, cypria, syriaca, caucasica, meda*;
*Line C - *Apis mellifera* subspecies Central and Southern European Group includes the *Apis mellifera*: *sicula, ligustica, carnica* (the Carniolian bee), *macedonica, cecropia*;
***Line M - *Apis mellifera* subspecies western and northern European Mediterranean group includes the *Apis mellifera*: *mellifera, iberica* subspecies.

Table 5. Analyzed wings sample from Cocon - Calarasi County

Wing	Line	Cocon - Calarasi									
		Carni	ca	Ligus	tica	Ceer	opia	Buck	fast	Melli	fera
1	C	89		21							
2	C	90				5		11			
3	C	90		11							
4	C	98						16			
5	C	92		9							
6	C	90		15							
7	C	91						4			
8	C	87		16							
9	C	99						8			
10	C	96		3						5	
11	C	90		12							
12	C	91								6	
13	C	92		9						7	
14	C	94									
15	C	95		12,34							
16	C	90		17				6			
17	C	91		7							
18	C	97								12	
19	C	88		14,89							
20	C	93		16							

Wing	Line	Cocon - Calarasi									
		Caucasica		Cypria		Anatolica					
1	C		2								
2	C										
3	C										
4	C										
5	C										
6	C										
7	C		3								
8	C										5
9	C										
10	C										
11	C										
12	C										7
13	C										
14	C										
15	C										
16	C										
17	C										9
18	C		12								
19	C										
20	C										

**Line O - *Apis mellifera* subspecies the Middle East group includes the *Apis mellifera*: *anatolica, adami, cypria, syriaca, caucasica, meda*;
*Line C - *Apis mellifera* subspecies Central and Southern European Group includes the *Apis mellifera*: *sicula, ligustica, carnica* (the Carniolian bee), *macedonica, cecropia*;
***Line M - *Apis mellifera* subspecies western and northern European Mediterranean group includes the *Apis mellifera*: *mellifera, iberica* subspecie

- in Giurgiu county, 16 wings from 20 showed a homology over 90% with *Apis mellifera carnica* (see Table 6);

Table 6. First analyzed wings sample from Gostinu - Giurgiu county

Wing	Line	Gostinu - Giurgiu							
		Carnica	Ligustica	Cecropia	Buckfast	Mellifera	Caucasica	Cypria	Anatolica
1	C	89	23			14,34			
2	C	90		2			21,05		
3	C	92	10		7				
4	C	90				11	6		
5	C	90				12	8		4
6	C	91				22			
7	C	96,12	11						
8	C	88			25				3
9	C	93,21					11		2
10	C	98	12						
11	C	98	11,2			12			
12	C	98			10				1
13	C	95		3			12		
14	M	12				62,33			
15	C	90	16						
16	C	88				21			
17	C	94					6		
18	C	97,11	2						
19	C	95	9		6				
20	C	94			1	5			

**Line O - *Apis mellifera* subspecies the Middle East group includes the *Apis mellifera*: *anatolica, adami, cypria, syriaca, caucasica, meda*;
*Line C - *Apis mellifera* subspecies Central and Southern European Group includes the *Apis mellifera*: *sicula, ligustica, carnica* (the Carniolian bee), *macedonica, cecropia*;
***Line M - *Apis mellifera* subspecies western and northern European Mediterranean group includes the *Apis mellifera*: *mellifera, iberica* subspecies.

- in Olt county, the sample Visina could not be classified in any subspecies, most wings expressing variable relatedness to one of the subspecies, without being possible to assess the type or level of hybridization (see Table 7);the sample from Bals (18 wings from 20) presented a homology over 90% with *Apis mellifera carnica* (see Table 8);

Table 7. Analyzed wings sample from Visina - Olt county

Wing	Line	Visina - Olt							
		Carnica	Ligustica	Ceeropia	Buckfast	Mellifera	Caucasica	Cypria	Anatolica
1	C	35					20		21
2	C	99	6						
3	C	51,71	43						
4	C	98,05					3		
5	C	72					23		7
6	O		18				5		83,52
7	C	61,91	11						
8	C	79	19	14					
9	C	79,45				14			3
10	C	59					33		
11	C	48	3	7			30		
12	C	65	2,01				30,35		
13	C	72,95	23						3,56
14	C	47			17	12			
15	C	90					11		
16	C	69	21	11					
17	C	52					33		
18	C	77	15,01		3				
19	C	83	8		7				
20	O			19			70		

**Line O - *Apis mellifera* subspecies the Middle East group includes the *Apis mellifera*: *anatolica, adami, cypria, syriaca, caucasica, meda*;
*Line C - *Apis mellifera* subspecies Central and Southern European Group includes the *Apis mellifera*: *sicula, ligustica, carnica* (the Carniolian bee), *macedonica,cecropia*;
***Line M - *Apis mellifera* subspecies western and northern European Mediterranean group includes the *Apis mellifera*: *mellifera, iberica* subspecies.

Table 8. Analyzed wings sample from Bals - Olt county

Wing	Line	Bals - Olt										
		Carni	ca	Ligus	tica	Ceer	opia	Buck	fast	Melli	fera	Cauc	asica	Cypri	a	Anat	olica
1	C		90									12					
2	C		90						8			10					
3	C		98		7												
4	C		97								22						
5	C		98		15,47												
6	C		89		16,29				4								
7	C		94									3,87					
8	C		93				8				11						
9	C		93								3,44						4
10	C		90				1				8						
11	C		98								13,4						
12	C		97									15					
13	C		99									20,55					
14	C		90		18												
15	C		90								10	5					
16	C		91									19					
17	C		96									23					
18	C		96						8,48			3					
19	C		97		13												
20	C		88				9										12

**Line O - *Apis mellifera* subspecies the Middle East group includes the *Apis mellifera*: *anatolica, adami, cypria, syriaca, caucasica, meda*;
*Line C - *Apis mellifera* subspecies Central and Southern European Group includes the *Apis mellifera*: *sicula, ligustica, carnica* (the Carniolian bee), *macedonica, cecropia*;
***Line M - *Apis mellifera* subspecies western and northern European Mediterranean group includes the *Apis mellifera*: *mellifera, iberica* subspecies.

- in Dambovita county: the samples from Gura Ocnitei (17 wings from 20) and Pucioasa (17 wings from 20) presented a homology over 90% with *Apis mellifera carnica* (see Tables 9 and 10);

Table 9. Second analyzed wings sample from Gura Ocnitei - Dambovita county

Wing	Line	Gura Ocnitei - Dambovita							
		Carnica	Ligustica	Ceeropia	Buckfast	Mellifera	Caucasica	Cypria	Anatolica
1	C	99	9						
2	O					12			89
3	C	90	6						
4	O		2		5				77
5	C	92	17			1			
6	C	93			4			3	
7	C	97	7		2				
8	C	91	1				2		
9	C	96				5,6			
10	C	96			9				
11	C	98			7				
12	C	98	9						
13	C	91		2				7	
14	O		7			1			79
15	C	91					10		
16	C	96				12			
17	C	91			12			2,07	
18	C	91	11						
19	C	93	9		10				
20	C	90	8,21		3				

**Line O - *Apis mellifera* subspecies the Middle East group includes the *Apis mellifera*: *anatolica, adami, cypria, syriaca, caucasica, meda*;
*Line C - *Apis mellifera* subspecies Central and Southern European Group includes the *Apis mellifera*: *sicula, ligustica, carnica* (the Carniolian bee), *macedonica,cecropia*;
***Line M - *Apis mellifera* subspecies western and northern European Mediterranean group includes the *Apis mellifera*: *mellifera, iberica* subspecies.

Table 10. Analyzed wings sample from Pucioasa - Dambovita county

Wing	Line	Pucioasa - Dambovita							
		Carnica	Ligustica	Ceeropia	Buckfast	Mellifera	Caucasica	Cypria	Anatolica
1	C	90							
2	C	90	22						
3	C	97	11						
4	O					17			88
5	C	90			5	12			
6	C	93					8		
7	C	98			11				
8	C	90	1		6				
9	C	89				5,6	12		
10	C	95					3		
11	C	97,1			6				
12	C	91	12						
13	C	91		5				10	
14	C	96			8,01				
15	C	88			1		14		
16	C	91,88				16			
17	C	94						3	
18	C	99						12	2
19	C	98			10				
20	C	95	8		1				

**Line O - *Apis mellifera* subspecies the Middle East group includes the *Apis mellifera*: *anatolica, adami, cypria, syriaca, caucasica, meda*;
*Line C - *Apis mellifera* subspecies Central and Southern European Group includes the *Apis mellifera*: *sicula, ligustica, carnica* (the Carniolian bee), *macedonica,cecropia*;
***Line M - *Apis mellifera* subspecies western and northern European Mediterranean group includes the *Apis mellifera*: *mellifera, iberica* subspecies.

- in Ialomita county, 17 wings from 20 showed a homology over 90% with *Apis mellifera carnica* (see Table 11);
- in Prahova county, the samples from Sinaia and Breaza (19 wings from 20) presented a homology over 90% with *Apis mellifera carnica* (see Tables 12 and 13).

Table 11. Analyzed wings sample from Marsilieni - Ialomita county

Wing	Line	Marsilieni - Ialomita							
		Carnica	Ligustica	Cecropia	Buckfast	Mellifera	Caucasica	Cypria	Anatolica
1	C	98	7						
2	C	90	25						
3	C	92	20						
4	O					26		11	68
5	C	89				18			21
6	C	91,38					6		
7	C	90	11		7				
8	C	98,98	0,3						
9	C	91					7		2
10	C	96,6	9						
11	C	92			4				
12	C	93	15				3		
13	C	90		2					10
14	C	91,3			9				
15	C	98					3		
16	C	91,98				12			
17	C	96,42						6	
18	C	91	1						9
19	C	89,67			13				
20	C	97	2	4					

**Line O - *Apis mellifera* subspecies the Middle East group includes the *Apis mellifera*: *anatolica, adami, cypria, syriaca, caucasica, meda*;
*Line C - *Apis mellifera* subspecies Central and Southern European Group includes the *Apis mellifera*: *sicula, ligustica, carnica* (the Carniolian bee), *macedonica,cecropia*;
***Line M - *Apis mellifera* subspecies western and northern European Mediterranean group includes the *Apis mellifera*: *mellifera, iberica* subspecies.

Table 12. Analyzed wings sample from Sinaia Prahova county

Wing	Line	Sinaia - Prahova							
		Carnica	Ligustica	Cecropia	Buckfast	Mellifera	Caucasica	Cypria	Anatolica
1	C	98,6							
2	C	93	12						
3	C	95	11						
4	O					2,17		23,15	71,51
5	C	91,38				16,68			21,77
6	C	90,24					1,45		
7	C	98			1,56				
8	C	98			0,2				
9	C	97					5,6		1,02
10	C	92							
11	C	91			3,4				
12	C	90					11,9		
13	C	94		3,04					10,7
14	C	91,3			7				
15	C	94					9,11		
16	C	91,01							
17	C	95					11,22		
18	C	91							12
19	C	90			11,56				
20	C	92,12		8,23					

** Line O - *Apis mellifera* subspecies the Middle East group includes the *Apis mellifera*: *anatolica, adami, cypria, syriaca, caucasica, meda*;
*Line C - *Apis mellifera* subspecies Central and Southern European Group includes the *Apis mellifera*: *sicula, ligustica, carnica* (the Carniolian bee), *macedonica,cecropia*;
***Line M - *Apis mellifera* subspecies western and northern European Mediterranean group includes the *Apis mellifera*: *mellifera, iberica* subspecies.

Table 13. Analized analyzed wings sample from Breaza - Prahova county

Wing	Line	Breaza - Prahova							
		Carnica	Ligustica	Cecropia	Buckfast	Mellifera	Caucasica	Cypria	Anatolica
1	C	95							
2	C	93,11	22						
3	C	91	9,8						
4	O					1,87			73
5	C	90,2				17			
6	C	93					3,89		
7	C	93			2				
8	C	98,04	0,2						
9	C	94				5,6			
10	C	96,44							
11	C	97,1			3,4				
12	C	94,11	11,3						
13	C	90		5,89				10,67	
14	C	91,65			8,01				
15	C	90					9		
16	C	91,88				16			
17	C	95						2,07	
18	C	90						10,98	
19	C	90			12				
20	C	93,14			2,43				

**Line O - *Apis mellifera* subspecies the Middle East group includes the *Apis mellifera*: *anatolica, adami, cypria, syriaca, caucasica, meda*;
*Line C - *Apis mellifera* subspecies Central and Southern European Group includes the *Apis mellifera*: *sicula, ligustica, carnica* (the Carniolian bee), *macedonica, cecropia*;
***Line M - *Apis mellifera* subspecies western and northern European Mediterranean group includes the *Apis mellifera*: *mellifera, iberica* subspecies.

CONCLUSIONS

Based on the results of geometric morphometry upon the analyzed wings, the honey bee populations studied has hybrids but also pure breed identified as being *Apis mellifera carnica* based on the homology over 90%. The pure breed were identified in samples originating from Calarasi (Cocon), Giurgiu, Olt (Bals), Dambovita (Gura Ocnitei, Pucioasa), Ialomita and Prahova (Sinaia and Breaza).

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