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PRELIMINARY STUDY OF *C. (MORPHOCARABUS) ZAWADZKII SERIATISSIMUS* REITTER 1896 FROM NORTHERN ROMANIA

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Abstract: *The study carried on C. (Morphocarabus) zawadzkii Reitter 1896, taxon belonging to the controversial supraspecific level: subsp. of C. (Morphocarabus) scheidleri Panz.; subsp. of C. (Morphocarabus) zawadzkii Kr. In a forthcoming paper carrying on 8 species from Romania and 1 from France, the marker 28Sr ensures the next regrouping: I. the super-group rothi with hampei, rothi, comptus, alutensis, kollari where C. (Morphocarabus) kollari Palliardi should be attached at rothi and not at scheidleri; II. group seriatissimus-incomptus, not positioned in relation to zawadzkii and scheidleri because of samples lack; III. monilis et excellens in separates groups. However the endophallus of seriatissimus has a close form to that of C. (Morphocarabus) zawadzkii dissimilis Csiki but different from that of C. (Morphocarabus) scheidleri Panz. to which, without doubt, seriatissimus is not attached. The geographical distribution in Romania (RO) and in Ukraine (UA) was clarified (bibliographic data of variable ancientness and recent collections). The known localities (especially RO) appearing on a map. The species occurs in the Maramureş Mountains (Romania, Ukraine), Podpula (UA), Ţibleş Mountains (RO) and western foothills of Rodna Mountains (RO). It cohabits with C. (Morphocarabus) incompsus Kr. 1880 in the Chornogora Mountains (UA) and perhaps in the Tibleş Mountains (RO). An analysis concerns two populations groups: the north zone (Lunca la Tisa, Bocicoel, Repedea, Ruscova, Borşa) in the Maramures County and the east zone (Romuli, Telciu) at western edge of the Rodna Mountains in the Bistriţa Nasaud County. The morphometric characteristics distinguish these two groups: the individuals of the East group have higher dimensions to those of the North group. The morphometric analysis was completed with the molecular biology analysis. The molecular markers COI I, cytochrome c oxidase subunit I and 16 S rRNA clearly distinguish the northern and eastern groups with a modest variability (differences 6 base pairs for the COI I, 5 base pairs for cytochrome b), either respectively an interpopulation variability of 1.15 and 0 75%; compatible values with the literature data. The 12 S marker does not provide any distinction. In the analysis of COI I some origins of C. (Morphocarabus) incompsus Kr. (species belonging to the same group as C. (Morphocarabus) zawadzkii seriatissimus Reitter) ranks near the eastern group. The nuclear markers Wg and Pepck show the sequence positions with individuals supernumerary holding bases whose origin is discussed (insufficient samples, introgression). A possible introgression requires the study of more individuals and to identify a possible donor.*

Keywords: *Carabus (Morphocarabus) zawadzkii seriatissimus; taxonomy, phylogeny, molecular biology*

I. Origin of the type. Geographical distribution.

In 1896, Reitter described very briefly a form of *seriatissimus zawadzkii* from a specimen of large size (39 mm) captured near Maramoros in an unspecified locality. In 1906, Csiki completed this primary description and mentioned the presence of this species only in the Northern part of Komitat Maramures. At that time, this territorial subdivision depended on the Austro-Hungarian Empire and included the Ukrainian territory beyond the river Tisa which marks the current border between Romania and Ukraine. In 1946, Csiki mentions the species around Kőrösmező (currently Jasinia) Ukrainian Maramureș, probably corresponding to the place of the original type described by Reitter.

In 1965, Mandl described *Carabus scheidleri n. maramorschensis* nat. nov. This description correspond to specimens called "Brenske" (not Reitter) but that are not different according to the description and the geographical origin of *seriatissimus* Reitter. Thus, *maramorschensis* is a synonym of *seriatissimus*.

Before the description of *seriatissimus* by Reitter, the corresponding species, *zawadzkii*, was mentioned in Romania (Bieltz 1852, Fuss 1871-1873) from Tibleș Mountains (Maramureș County), the gold mines of Lapuș, Băița, Baiuț, Targu Lapuș. In 1932, Breuning considers *seriatissimus* as an individual sculptural variant and reports its presence in the following localities: Sighetu Marmației and Munții Rodnei in Romanian and the Cerna Hora (winter sports resort of the massif Hoverla from the Chornohora Mountains) in Ukraine. More recently other areas were confirmed or discovered (see map, figure 1).

a. In Romania

Maramureș County : Sighetu Marmației, Dobăieș (MERK 2008) and the nearby areas Bocicoiu Mare (KINGA *et al.* 2003) ; Valea Izei and Dealu Solovan (Natura 2000), Lunca la Tisa (BARLOY *et al.* 2007, 2011), Vișeu de Sus (TAKACS 2002, 2003), Repedea et Ruscova (Monts Maramures), Bocicoel et Bogdan Voda (BARLOY et al 2011), Strambu Băiuț in the Gutâi Mountains (Natura 2000), Borșa 920 m.

Bistrița Năsăud County: western edge of the Rodna Mountains (Sălăuța Valley, Romuli, Fiad, Telciu (BARLOY *et al.* 2011).

b. In Ukraine

In addition to the localities cited by Csiki (1906) and BREUNING (1932), must to add those mentioned by par PANIN (Carabidae of the World): Bogdan (Mt. Chornogora), Dilove (Mt. Maramures), Svidovec (Mt. Podpula).

● **Confirmed stations: Bc-Bocicoel, Bg-Bogdan, Bm-Bocicoiu Mare, Bo-Borșa, Bv-Bogdan Vodă, Di-Dilowe, Fi-Fiad, Lt-Luca la Tisa, Re-Repedea, Ro-Romuli, Ru-Ruscova, Ti-Telciu, Vs-Vișeu de Sus**

● **Suspected stations: Ba-Băiuț, La-Lăpuș, Sb-Strâmbu Băiuț, Su-Suplai, Tl-Târgu Lăpuș, Vi-Valea Izei**

Figure 1. Distribution of *C. (Morphocarabus) zawadzkii seriatissimus* northern Romania

The presence of the species is well established in Ukraine (east of southern Ukrainian Carpathians) and Romania (Maramureș Mountains; probably Tibleș Mountains and Western Mountains Rodna). The studied populations come from the following localities: Lunca la Tisa : 47^{0}56 N, 24^{0}02 E, alt. 320 m. ; Ruscova et Repedea : 47^{0}49 N, 24^{0}21 E, alt. 500 m. ; Bocicoel : 47^{0}42 N, 24^{0}18 E, alt. 481 m. ; Borșa: 47^{0}37 N, 24^{0}48 E, alt. 920 m. ; Romuli: 47^{0}32 N, 24^{0}25 E, alt. 670 m. ; Telciu: 47^{0}26 N, 24^{0}24 E, alt. 550 m.

II. Taxonomic aspects

The attribution of the taxon *seriatissimus* Reitter at a supraspecific level is controversial and could be considered: (1) as subsp. of *C. (Morphocarabus) scheidleri* by Breuning (1932) in fact *monilis (scheidleri)*; Kryzhanovski *et al.* 1995, Brezina 1999, 2001, Kleinfeld *et al.* 1999 recognizing only *zawadzkii* or (2) as subspecies of *C. (Morphocarabus) zawadzkii* Kraatz 1885 by Reitter (1896), Csiki 1905-1946, Turin *et al.* 2003, Deuve 2004 or (3) as the correct species by Lobl *et al.* 2003, but attached to *scheidleri*.

Based on molecular analysis results (see below), the kinship with monilis should be excluded but some studies (Szel 2007, pers.) shows that the Hungarian *zawadzkii* is very close to some forms of *C. (Morphocarabus) scheidleri* as *vertesensis* Retezar 1974 (moreover uncertain taxon). The dominant presence (see below) of 4 primary intervals to the elytra, characteristic to *zawadzkii,* advocates for the incorporation of *C. Morphocarabus zawadzkii* to *seriatissimus*.

III. Morphology and morphometric characteristics

All studied populations (see below) are black, females have very mat elytra at where the brightest males have often a greenish white reflect. The edges of the elytra and the pronotum have a dominant blue or a blackish blue color.

a. Primary intervals

In principle, the elytron of C. *(Morphocarabus) zawadzkii* has 4 primary intervals and *C. (Morphocarabus) scheidleri* only 3. For *seriatissimus*, elytra are very flat and have intervals indicated by lines of points or very short dashes, always very superficial. The primary intervals, not splitted, can be more or less identified by the presence of circular shallow fovea, having a acute brilliant mucron in the front part of the depression. This line of fovea can be found on a rather broad elytra band, slightly raised from the rest of the elytra, which facilitates its retrieval.

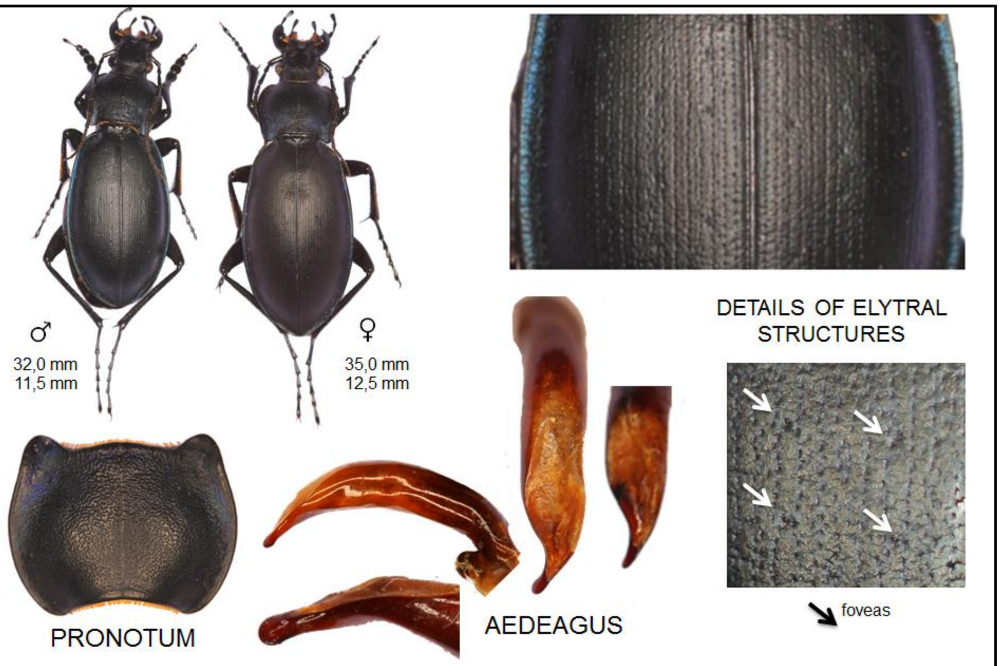


Figure 2. *C. (Morphocarabus) zawadzkii seriatissimus* Reitter 1896. Repedea – Maramureș

The foveas are in generally rare and very superficial on the disk (1^0 and 2^0 primary) but more numerous and deep on the other intervals and in the posterior part. The primary interval is not always detectable:

-absence or erasure on the elytra disc,

-fourth outer primary undetectable, because doubtless, encompassed in the granules of the elytra edge.

- The examination of individual's important effective shows a variability of the number of primary identifiable intervals:

-4 primary at 59% of male insects, 54% of female insects

-3 primary in 27% of male insects, 33% of female insects; the fourth primary being indistinguishable or the first primary being absent

-13-14% of individuals have no detectable primary interval, the elytra being totally flat.

The examination of a large effective shows a variability of the number of identifiable primary intervals: 4 primary for 59% of the males and 54% for the females; 3 primary in 27% of the males and 33% for the females; the fourth primary being indistinguishable or the first primary being absent and 13-14% of specimens have no detectable primary interval, the elytra being totally flat. The variability of the number of primary intervals compare to the reference value of the species type had already been mentioned by LAPOUGE (1916) for various species of *G. Morphocarabus* of therefore *zawadzkii*: this phenomenon being frequent in Transylvania and in Carpathians. This anomaly has not been evaluated because it requires to collect large series. PANIN (2013) mentions the presence of 4 primary intervals at only 20 to 30 % into a population of *C. (Morphocarabus) zawadzkii* s str.

- The intervals II and III, indistinguishable each other, subdivided into length, form lines or fragments of lines, more or less completes and vague points or dashes.

At high magnification, the intervals flat on the disk, more or less convex on the sides (especially for the anterior and posterior parts) are striped across by lines that delimit variable convex segments, the granulation of fund being very shallow.

b. Morphometric measurements

The measurements and the statistical analysis concerns the 6 studied populations, Romuli and Telciu having identical populations.

Table 1.

Some morphometric characteristics of *C. (Morphocarabus) zawadzkii seriatissimus* Reitter 1896

	Size (mm)			Ratio			Pronotum	
♂	**L**	**l**	**Le**	**L/l**	**Le/L$_{\%}$**	**Le/l**	**e/h**	**h/e**
Repedea	32,15	11,84	19,92	2,71	62,00	1,68	1,39	0,72
Borșa	33,01	11,20	20,26	2,93	61,40	1,80	1,34	
Lunca	33,20	12,07	21,26	2,75	64,04	1,76	1,44	0,69
Bocicoel	34,56	12,19	20,22	2,83	61,41	1,74	1,44	0,69
Romuli-Telciu	37,75	1350	24,00	2,80	63,58	1,77	1,50	0,67
♀	**L**	**l**	**Le**	**L/l**	**Le/L$_{\%}$**	**Le/l**	**e/h**	**h/e**
Repedea	35,19	12,60	21,67	2,79	61,56	1,72	1,40	0,71
Borșa	35,16	12,70		2,76	64,01	1,76	1,51	
Lunca	36,20	12,77	22,64	2,83	62,55	1,77	1,51	0,66
Bocicoel	36,72	12,91	23,00	2,84	62,63	1,78	1,47	0,68
Romuli-Telciu	39,80	14,33	24,70	2,78	62,06	1,72	1,45	0,69

L: maximal length; l: elytra maximal width; Le: elytra maximal length; e: maximal pronotum width; h: maximal pronotum height in the central part

Measures on 30♂♂ and 30 ♀♀ of every population

In these localities, some female are 42 to 44 mm in length for 15.0 to 15.5 mm wide.

The populations have notable differences of size, without strict relation with the altitude of the place. Certain females from Romuli-Telciu achieve or exceed 41 mm long and 14 to 14,5 mm wide. The specimens of the populations of the East group have statistically dimensions superior of those of the North group.

c. Shape of the endophallus

The picture of the endophallus of *C. (Morphocarabus) zawadzkii seriatissimus*, from Lunca la Tisa, the typical area of this species, has a similar the shape as the of *C. (Morphocarabus) zawadzkii ssp* rather than that of *scheidleri*.

Carabus (Morphocarabus) zawadzkii seriatissimus Reitter 1896
Lunca la Tisa (Romania)

Carabus (Morphocarabus) zawadzkii dissimilis Csiki, 1906
Uzhhorod (Ukraine)

Carabus (Morphocarabus) zawadzkii zawadzkii, Kraatz 1854
Zelemjanka (Ukraine)

Carabus (Morphocarabus) scheidleri scheidleri Panzer, 1799
S. Bohemia (Czech Republic)

Figure 3. Endophallus of *Carabus (Morphocarabus) zawadzkii* (photos Panin Ruslan)

IV. Molecular biology

IV. 1. Material

The studied specimens originate from the populations of the Northern group: Lunca la Tisa, Repedea, Bocicoel and from the eastern group: Romuli-Telciu (geographic coordinates see paragraph I).

IV. 2. Methods

The insects, harvests in traps after 2 at 4 days of stay, are placed in ethanol 95° then stored at 20° C. The 2 femurs were collected and manually crushed. The crushing is then transferred in 180 µl of proteinase K buffer solution and 20 µl proteinase K enzyme and incubated overnight at 56^{0} C. The DNA was then recovered using Qiagen QIAamp mini kit following the manufacturer recommendations. Washing was made according to the Qiagen protocol and 2 successive elutions were done with 50 µl H_2O then 25 µl H_2O (total 75 µl). DNA was quantified by spectrophotometry.

- *PCR realization*

PCR with 2 µl ADN with 30 µl fluid with Taq type it+cycles:

1^0 ·96 ^{0}C, 4 min. /40 cycles at: 96^0C, 1 min/TM 1, min/72^0C, 1 min.
/45 cycles for Cyt b (CP_1 –CB_2)
·72 ^{0}C, 4 min. then hold at 15^0C before purification

·TM used : -Cyt b. (CP_1 –CB_2) : 46^0C
-COI I (LCO1490, HCO2198) : 48^0C
-16S (LRJ12887 mod-LRN13398: 50^0C
-12S (SRJ14233-SRN14588): 52^0C
-Pepck (CARPEK1-CARPEK3): 55^0C
-Wg (carwl1-carwl2): 58^0C
-28S(28SO1-28RO1): 58^0C
-*Anonymous* (gwenek1, carck2): 60^0C

2^0 Purification 10 µl extract PCR on column G50
Sequence reactions
·96^0C, 4 min/96^0-45 sec/32 cycles TM according to markers 45 sec./62^0C, 45 sec.
·TM idem first cycle
Hold at 15^0C before sequencing

3^0 Migration on sequencer 3130 XI Genetics Analyser Applied Biosystems.
b. Primers. cf. list in Deuve *et al.* 2012.
c. Sequence analysis

Sequences were analyzed using the software Geneious version 7. Multiple alignments ware performed with the algorithm MAFFT (Katoh 2002) and the phylogenetic trees were made using the algorithm PHYML (Guindon 2010) with the substitution model IC69 and a bootstrap of 100 replicates.

IV. 3. Results

A. Marker 28S rRNA.

The global analysis on the *Morphocarabus* group was made using 15 origins of *hampei,* 13 origins of *rothi*, 6 origins of *seriatissimus*, 3 origins of *alutensis*, 3 origins of *comptus,* 2 origins of *incompsus*, 2 origins of *kollari*, 1 origin of *excellens* et 5 origins of *monilis,* the latter 5 from Southern and South-eastern of France. The analysis shows the existence of 4 groups:

I. group *rothi* with *rothi; hampei; alutensis; comptus; kollari*
II. group *seriatissimus-incompsus*
III. group *excellens*
IV. group *monilis*

These groups differ between them by 3 pairs of base (on 558 nucleotides)

Table 2.

Differences revealed by global analysis

Nucleotide	**38**	**106**	**243**
Group I	A	G	A
Group II	T	G	A
excelens	T	G	G
monilis	T	A	A

It follows that *kollari*, related until now to *Sg. scheidleri*, belongs to the *rothi* group. Due to the lack of specimens belonging to *C. (Morphocarabus) scheidleri* and *C. (Morphocarabus) zawadzkii*, the connection of Group II at supraspecific level was impossible. However a study realized by a hungarian team shows a certain closeness between *C. (Morphocarabus) zawadzkii* and *C. (Morphocarabus) scheidleri vertensis* Retezar (pers. com. SZEL 2007).

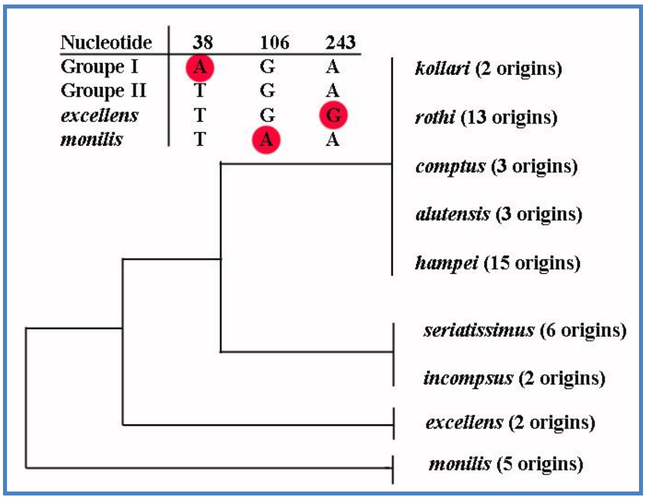


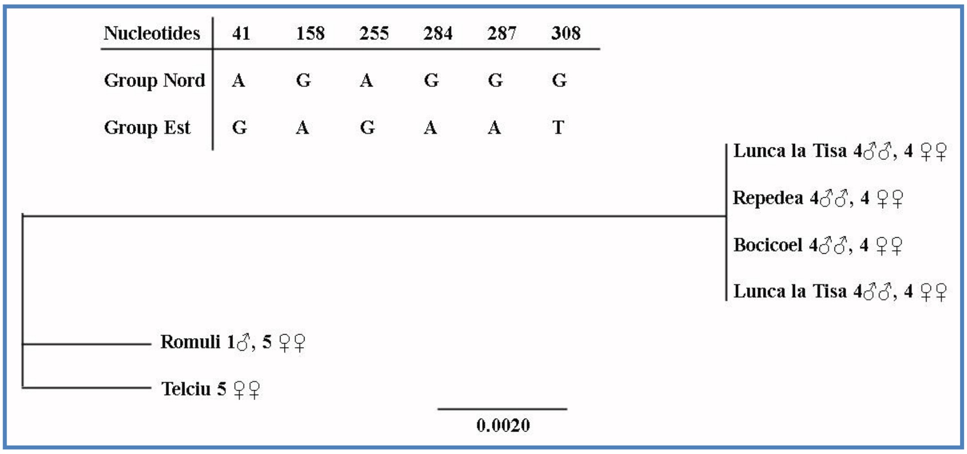
Figure 4 Molecular global analysis on the *Morphocarabus* with 28S rRNA marker

B. Molecular markers

1. Analysis with CoI I marker

The analysis of the results shows two different groups that differ from 6 nucleotides (on 523 nt) i.e 1.15% of variability.

A female exemplar from Telciu differ from all the others in the site 45 where a T base instead of a C base.



Nucleotides	41	158	255	284	287	308
Group Nord	A	G	A	G	G	G
Group Est	G	A	G	A	A	T

Figure 5 Molecular analysis with CoI I marker

The suspected presence of *C. (Morphocarabus) incompsus* in the zone of Bichigiu, 10 km West of Romuli, incited us to introduce, into the analysis, specimens from Bogata, a very distant locality, in order to estimate the position of this species that 28S tree relate to *seriatissimus*.

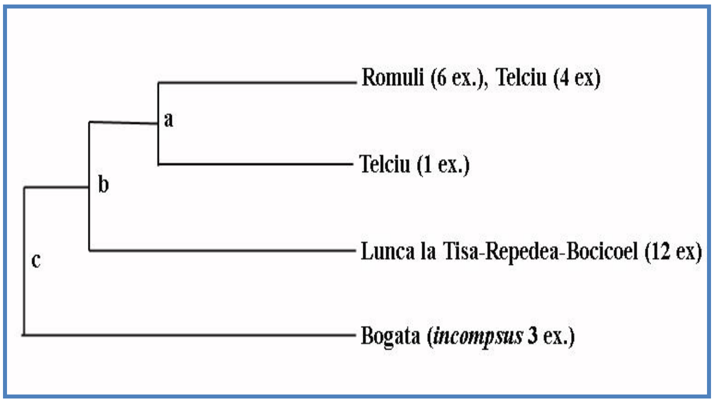
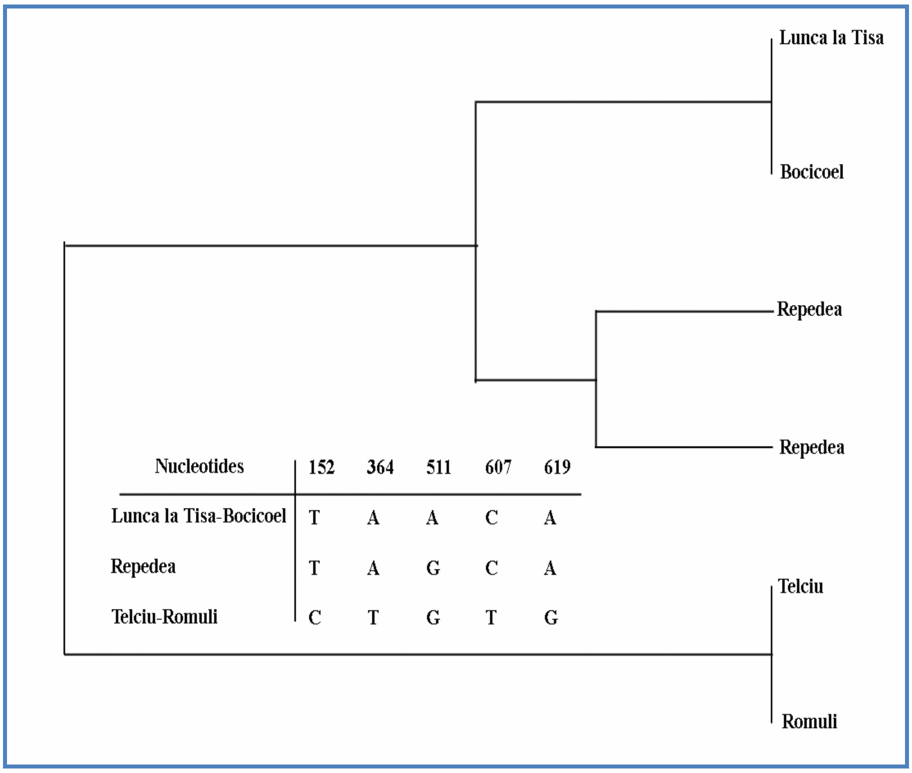


Figure 6 Differences in base pair with CoI I marker

The population of *incompsus* seems heterogeneous, nevertheless, a male is close to the East group (differences: 3 nt); differences in base pair (a=1, b=7, c=14). To decide on the degree of closeness *seriatissimus-incompsus*, it would be necessary to have populations from both North and East zones.

2. Analyse with *cyt b* marker

As for CoI I, *cyt b* differentiates North and East groups, the differences concerning 5 nt (on 684 nt) i.e 0.7%. It is possible that Repedea des Monts Maramureș being different from Lunca la Tisa and Bocicoel.



Nucleotides	152	364	511	607	619
Lunca la Tisa-Bocicoel	T	A	A	C	A
Repedea	T	A	G	C	A
Telciu-Romuli	C	T	G	T	G

Figure 7 Differences in base pair with *cyt b* marker

3. Analyse with 12S marker

This marker does not indicate any difference between the populations whatever their origin.

4. Analyse with 16S marker

This marker indicates, such as *cyt b*, one difference of base (position 240: A group East; G group North); a point mutation that may have occurred to an individual of Bocicoel (354 base T link to A) and another of Lunca la Tisa (485, base T link to C).

C. Nuclear markers

1. Marker Wg

The analysis concerns 7 specimens from the East zone (Romuli-Telciu) and 12 from the North zone (Repedea-Lunca la Tisa-Bocicoel). Based on the results obtained with cytoplasmic markers, these specimens seem to be representative of each population origins. The results indicate differences in population structure in 5 positions: 108-129-312-327-366; with identical bases in the zones East and North.

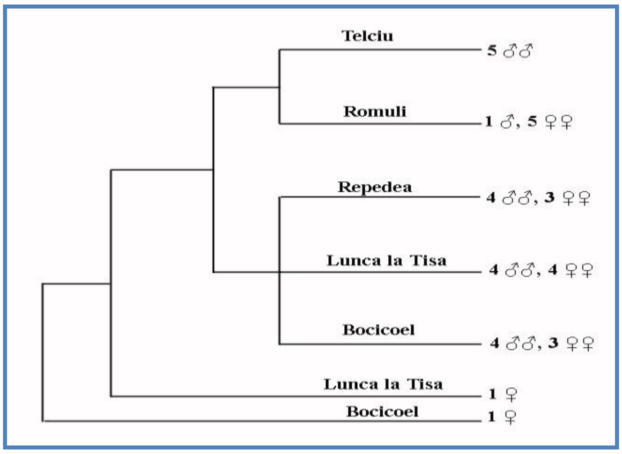


Figure 8 Differences in base pair with 16S marker

Table 2.

Differences revealed by Wg nuclear marker

	Position 108	Position 129	Position 312	Position 366	Position 327
Group E	$4H_{T}$ $3h_{CT}$	$3H_{A}$ $2H_{G}$ $2h_{AG}$	$4H_{A}$ $3h_{AG}$ 🟥🟩 G	$8H_{G}$ $1H_{T}$	$5H_{G}$ $1H_{A}$ $1h_{AG}$
Group N	$10H_{T}$ $1h_{CT}$ 🟥🟩 C	$6H_{A}$ $2H_{G}$ $4h_{AG}$	$6H_{A}$ $6h_{AG}$ 🟥🟩 G	$8H_{G}$ $2H_{T}$ $2h_{TG}$	$12H_{G}$

H : Homozygous ; h : heterozygous

This distribution raises problem for the position 108, where the C base is not represented and the position 312, where the G base is absent. This arises, maybe, from the low number of studied specimens; the missing bases being highly recessive. This does not exclude the hypothesis of the introgression for the each locus C and G. The increase of the number of studied individuals is necessary to confirm or refute one or the other of assumptions.

2. Marker Pepck

This marker indicates 22 different positions. If we make the analysis by zone; either 8 individuals East zone; 11 individuals North zone or the following arrangement:

Table 3.

Differences revealed by Pepck nuclear marker

	Position 8	Position 167	Position 173	Position 185	Position 200	Position 208	Position 212	Position 239	Position 251	Position 274	Position 303
Group E	$8H_{A}$ 🟥🟩 A	$3H_{A}$ $5h_{AT}$ 🟥🟩 T	$2H_{A}$ $1H_{G}$ $4h_{AG}$	$3H_{T}$ $3H_{G}$ $2h_{TG}$	$6H_{A}$ $1H_{G}$ 1_{AG}	$5H_{G}$ 2_{AG} 🟥🟩 A	$7H_{A}$ $1H_{G}$	$5H_{G}$ $1H_{A}$ $2h_{AG}$	$6H_{G}$ $1H_{A}$ $1h_{AG}$	$8H_{G}$	$7H_{G}$ $1h_{AG}$ 🟥🟩 A
Group N	$6H_{A}$ $5h_{AG}$ 🟥🟩 G	$2H_{T}$ $9h_{AT}$ 🟥🟩 A	$6H_{G}$ $1H_{A}$ $4h_{AG}$	$3H_{T}$ $2H_{G}$ $6h_{TG}$	$11H_{A}$	11_{G}	$8H_{A}$ $3h_{AG}$ 🟥🟩 G	$4\ H_{G}$ 7_{AG} 🟥🟩 A	$5H_{G}$ $6h_{AG}$ 🟥🟩 A	$8H_{G}$ $3h_{AG}$ 🟥🟩 A	11_{G}

	Position 317	Position 320	Position 355	Position 392	Position 416	Position 438	Position 452	Position 455	Position 476	Position 530	Position 536
Group E	$6H_{G}$ $2h_{AG}$ 🟥🟩 A	$8H_{G}$	$2H_{G}$ $2H_{A}$ 3_{AG}	$8H_{A}$	$2H_{G}$ $6h_{TG}$ 🟥🟩 T	$6H_{T}$ $2h_{CT}$ 🟥🟩 C	$6H_{G}$ $1H_{A}$ $1h_{AG}$	$7H_{A}$ $1h_{AG}$ 🟥🟩 G	$3H_{T}$ $1H_{C}$ $3h_{CT}$	$1H_{A}$ $2H_{G}$ $5h_{AG}$	$4H_{T}$ $1H_{A}$ $3h_{AT}$
Group N	11_{G}	$10H_{G}$ 1_{CG} 🟥🟩 C	$8H_{G}$ $3h_{AG}$ 🟥🟩 A	$9H_{A}$ $2h_{AG}$ 🟥🟩 G	$10H_{G}$ $1h_{TG}$ 🟥🟩 T	$10H_{T}$ $1h_{CT}$ 🟥🟩 C	$11H_{G}$	$11H_{A}$	$4H_{T}$ $7h_{CT}$ 🟥🟩 C	$9H_{G}$ $2h_{AG}$ 🟥🟩 A	$5\ H_{T}$ $1H_{A}$ $5h_{AT}$

🟩 Missing base

Considered separately, the populations [redacted] present a deficit in certain nucleotides. If we admit that the genetic structure of *C. (Morphocarabus) zawadzkii seriatissimus* is unique, the excess of one base arises for: the positions for the origin of the A base (8-208-239-251-274-303-317-355-530); the positions for the origin of the G base (8-212-392-455); the positions for the origin of the C base (320-438-476); the positions for the origin of the T base (167-416).

It asks the same question as for the Wg marker: the observed supernumerary bases is the result of an insufficiency of the number of studied individuals or the result of an introgression? the donor being an unidentified species.

V. CONCLUSIONS

The analysis of molecular biology shows that the discriminating cytoplasmic markers (*CoI 1*; *cyt b*; 16S) indicate a difference between the populations from the North (Lunca la Tisa, Bocicoel, Repedea) and those from the East in border of Rodna Mountains (Romuli-Telciu). Even if the variability remains modest (1,15 to 0,73%), it seems constant.

b. for nuclear markers (*Wg, Pepck)* and for a less important number of individuals, a high number of SNP (single nucleotide polymorphism) is observed but at this stage, it is difficult to conclude whether these variations are due to a low sampling or if it represents a introgression with a yet unidentified species. The position of *C. (Morphocarabus) incompsus* in taxonomic relation with *seriatissimus* remains doubtful and requires further studies.

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