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A New Subspecies of *C. (Eucarabus) obsoletus* Sturm 1815 (*Coleoptera: Carabidae: Carabini*) in Romania

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Abstract

C. (Eucarabus) obsoletus Sturm is a widespread species in Romania with a broad infra-specific diversification (8 subspecies mentioned). The morphological identification is difficult, constraining, requiring to observe a large number of insects with a result often random due to the insufficiency of criteria. The use of molecular biology with mitochondrial markers (COI I/cyt b concatenation), identified the genetic structure and the use of genetic distance (Matrix K2P Kimura) ensures classification and separation between groups.

The study applied successively this technique to the subspecies described, to the Czech and Slovak provenances, considered close to the type described by Sturm and to the 66 populations in Romania.The results with the subspecies described, collected from the localities indicated by their descriptor confirm the originality of *uhligi/csiki* (Rodna) *fossulifer/carpathicus/nagyagensis* but put in synonymy nagyagensis with *paranagyagensis-tippmani-prunneri*. The population of Moravia has the same genetic structure as csiki from Rodna Mountains, having a maternal origin no doubt neighbouring.The genetic analysis of the provenances of the territorial collection reports certain populations to the subspecies identified and reveals 5 additional groups: *obsoletus csiki* with 3 components (group *obsoletus csiki* (Rodnei Mountains) close to the type of Sturm, *csiki NW* (Lunca la Tisa) close to *uhligi*, *csiki* Lotrului Mt., Voineasa); group *obsoletus* Cozia, close to the previous ones and with a wide geographical distribution; *obsoletus* Parâng (and Poiana Rusca) are very different from all other groups with a genetic distance of 4.7 to 6.9% which constitutes a new subspecies.*Obsoletus* Parâng is a subspecies of alpine zone (type Parang Mountains 1000-2100 m) of small size (males L/l = 20.7/8.5 mm, females L/l = 21.5/9.1 mm) with the dominant two-coloured exemplars (pronotum brown red and elytra yellowish green or brownish, 18% melanizing at altitude). Morphologically, it belongs to groups of *obsoletus* at salient intervals, subequal with an entire interval after the third primary. The species has a very high infra-specific variability with genetic divergence rates between 3.0 and 5.6%.

In conclusion, for a species with intraspecific variability that is morphologically difficult to establish, the use of molecular biology (mitochondrial markers) allows the easy identification of subspecies and better accounts for biodiversity.

Keywords: Carabus obsoletus, subspecies, molecular biology, taxonomy, distribution, Parâng Mountains

Introduction

Due to morphological, chromatic, geographical, and genetic plasticity, an insect species frequently includes several subspecies, especially for those with wide geographical distribution.

The definition of a subspecies (subsp) is not exclusive and unanimously accepted: the subdivision of a geographically localized species distinguishing morphologically, genetically, sometimes biochemically from another subspecies and named according to taxonomic rules. In the past, the distinctive criteria were mostly morphological and ecological (localization in a biotope). Few studies have focused on the link between criteria cases and the genetic structure of insect subspecies. To our knowledge, no subspecies has been designated based on genetic and ecological characteristics.

Numerous interspecific genetic variability studies have been conducted mainly by the DNA barcoding method (COI I marker) and with other mitochondrial [1] or nuclear markers [2]; the works having sometimes been extended to the intraspecific genetic variability, some research being conducted on European Carabidae species [3, 4, 5, 6, 7, 8]. Inter and intra specific variabilities are often expressed by genetic divergence exploiting K2P Kimura parameter [9].

The paper is regarding to *C. (Eucarabus) obsoletus* Sturm 1815, a well-represented species in Romania but with a difficult morphological identification of the subspecies [10, 11].

The study carried out in molecular biology (concatenation COI I/cyt b) comprises two stages:

1. Characterization of subspecies identified by their descriptors from typical localities. This makes it possible to characterize each subsp. by a genetic reference structure.
2. Extension of the study to 64 populations of Romania and 3 populations of the Czech and Slovak Republics considered close to the type described by Sturm 1815.

Methodology

a. The insects captured with Barber traps (verified after 3-4 days) are preserved in alcohol 95^0 at -20 ^{0}C. For each locality (except those of the types) the analysis concerns 2 individuals (1 male and 1 female). In case of divergent results, which sometimes occur between male and female the number is 4 insects. The populations of the typical origins, collected in abundance, are treated for 6 individuals, (same number of males and females) and sometimes more, like for Sacaramb where, in the same place, two genetically different populations exist, but indistinct morphologically (20 exemplars studied).

Choice of markers:

In a preliminary study, trying to identify the most discriminating markers, these were not retained, because they are of little interest: 12S, 18S, Wg, ND1, ND4 and 28S.

Retained markers: two mitochondrial markers

- COI I: LCO1490; HCO_2 198, Hebert *et al*., 2003 [12];
- cyt b: CP1 [13]; CB_2 (Jermin & Crazier 1994).
- Method of material preservation, extraction, purification and sequencing, after Barloy *et al*., 2014.

b. Sequencing analysis

The results of the mitochondrial DNA sequencing are expressed in terms of genetic distance, as a percentage based on Kimura's K2P parameter [9].

Choice of a threshold value of intraspecific variability distinguishing subsp.

Indications of the literature:

Wangh [14] summarizing COI I works regarding many animal species, notes that intraspecific variation is usually less than 1%, rarely greater than 2%.

For the European*Carabidae*:

Raupach [5] for 21 *Bembidion* species note an intraspecific average divergence of 0.19% and a maximum of 1.92%.

Homburg [7] for 97 *Carabus (Platycarabus) irregularis* individuals, find a divergence of the intra-specific variability between 0.2 and 2.2%.

For the *Carabidae* from the other regions of the globe and for *Cincindelidae*, [15, 16] note a maximum intraspecific genetic distance of 2.00 to 2.25%.

Our results of intra-group genetic divergence variability show the following values (between populations with same genetic structure):

- *uhligi* 0.15; *Detunata* 0.42; *csiki Lotru* 0.45, *nagyagensis* 0.56; *paranagyagensis* 0.62; *csiki Rodna-csiki NE* 0.68; *csiki NW* 0.72; *carpathicus* 1.02; *Cozia* 1.06; *fossulifer* 1.15.

Taking into account of the data from the literature of the genetic divergence values intra (see above) and intergroups (see below), we use for the threshold value of distinction between subsp., a divergence of 2.2 to 2.3%.

Results and Discussion

Results for the type subspecies:

The table below lists the typical subspecies from the literature, their descriptors and their provenance from which the populations studied come from.

Table 1. Subspecies and localities of origin in Romania

Subspecies	**Type locality**	**Remarks**
-*csiki* Malasz 1900	Hardwood forests Rodnei Mountains (BN)	According to Petri 1912, it would be specimens supplied by Ganglbauer from the Rodnei Mountains.
-*uhligi* Holdaus 1910	Hăsmasul Mare (HR)	Short description completed by Breuning [18] under the name *mallaszianus*.
-*fossulifer* Fleischer 1893	Spinus (BH)	Village in Bihor County but not in Bihor Mountains (situated at 120 km SSE) as sometimes indicated. Spinus is located at 40 km NE from Oradea; Village at present devoid of forests.
-*nagyagensis* Seidlitz 1888	Sacaramb (HD)	Successive names= *carpathicus nagyagensis* (Birthler 1886), *procerus* (Bielz 1887) simplification by Seidlitz (1888) more or less accepted.
-*tippmanni* Breuning 1936	Zlatna (AB)	Beheim et Breuning [19] agree with a strong resemblance to *nagyagensis* Seidlitz.
-*prunneri* Malasz 1900	Detunata Mountains (AB)	Mallasz dedicates this taxon to R. Prunier, a mining engineer at Zlatna. Origin, Detunata Mountains, near Bucium[1].
-*paranagyagensis* Lie 1999	Forest of the hills of Lipaer=Lipova (HD) Pojoga	Non-admitted form, distinct from *nagyagensis* by the varied colour (brown, reddish brown, purple) against bluish black, the unique colour for *nagyagensis*.
-*carpathicus* Palliardi 1825		
-*euchromus* Palliardi 1825	Banat Mountains	Distinction between forms of: -lowland (250-300 m): *carpathicus* (L/l: 24-25/9-10 mm) -semi-mountain (1300-1400 m) *euchromus* (L/l: 21-22/8,2-8,5 mm).

-*bielzi* Birthler 1886	Cibin Mountains, Cindrel Mountains (SB)	Altitudinal form (1800-1900 m). Small size (L/l: 15-17/7-8 mm).
-*deubelianus* Fleischer 1911	Brasov Mountains (BR)	According to Csiki (1912) it would be an aberrant form.

[1] There are two mountains Detunata (Dyke basaltic andesite):
- Detunata Flocoasa (1265 m) with a coniferous forest up to the top,
- Detunata Goala (1108 m), denuded in the terminal part and housing at its base the taxon *prunneri* Malasz 1900, among the blocks of basalt.

The results for type subspecies

The table below shows the difference in genetic distance (percentage K80 Kimura matrix) differences between subspecies compared two to two.

Table 2. The genetic distance between the type subspecies in percent

	csiki	***uhligi***	***fossulifer***	***nagyagensis***	***paranagyagensis***	***tippmanni***	***prunneri***	***carpathicus***
csiki	-	(2.71)	(4.64)	(4.37)	(4.45)	(4.71)	(4.18)	(3.92)
uhligi	2.71	-	(4.32)	(3.94)	(4.10)	(4.54)	(3.91)	(3.52)
fossulifer	4.64	4.32	-	(4.71)	(5.24)	(5.21)	(4.44)	(4.52)
nagyagensis	4.37	3.94	4.71	-	(2.28)	(2.18)	(0.41)	(3.74)
paranagyagensis	4.45	4.10	5.24	2.28	-	(0.01)	(2.19)	(3.94)
tippmanni	4.71	4.54	5.21	2.18	0.01	-	(2.29)	(3.82)
prunneri	4.18	3.91	4.44	0.41	2.19	2.29	-	(3.69)
carpathicus	3.92	3.52	4.52	3.74	3.94	3.82	3.69	-

These results:
- identifying four different genetic structures: *csiki/uhligi/fossulifer/carpathicus*
- classify in the same group *nagyagensis-prunneri* and *paranagyagensis-tippmanni* (see the table below).

Table 3. The comparison between nagyagensis-prunneri and paranagyagensis-tippmanni (1100 bp)

	66	115	147	185	370	385	413	439	533	538	584	653	717	768	794	833	843	918	925	959	968	1067	1072	1075	1078
nagyagensis	A	T	C	C	T	A	T	T	T	A	T	T	C	G	T	A	A	A	A	G	T	C	A	A	G
prunneri	A	T	C	C	C	A	T	T	T	A	T	T	T	A	T	A	A	A	A	G	T	C	A	A	G
paranagyagensis	G	C	T	T	T	G	C	C	C	G	C	C	T	G	C	G	G	G	G	A	A	T	G	G	A
tippmanni	G	C	T	T	T	G	C	C	C	G	C	C	T	G	C	G	G	G	G	A	A	T	G	G	A

From a genetic point of view (Table 3, Fig. 1):

- *paranagyagensis* and *tippmanni* are identical,
- *nagyagensis* and *prunneri* are close, differing by three base pairs,
- *nagyagensis* and *paranagyagensis (tippmanni)* genetic distance = 2.28 different by 23 base pairs, mostly in substitution. Although located at the chosen significance level, we retain *paranagyagensis* as subsp, validation recognized by Deuve [20] but not by Lobl [21].

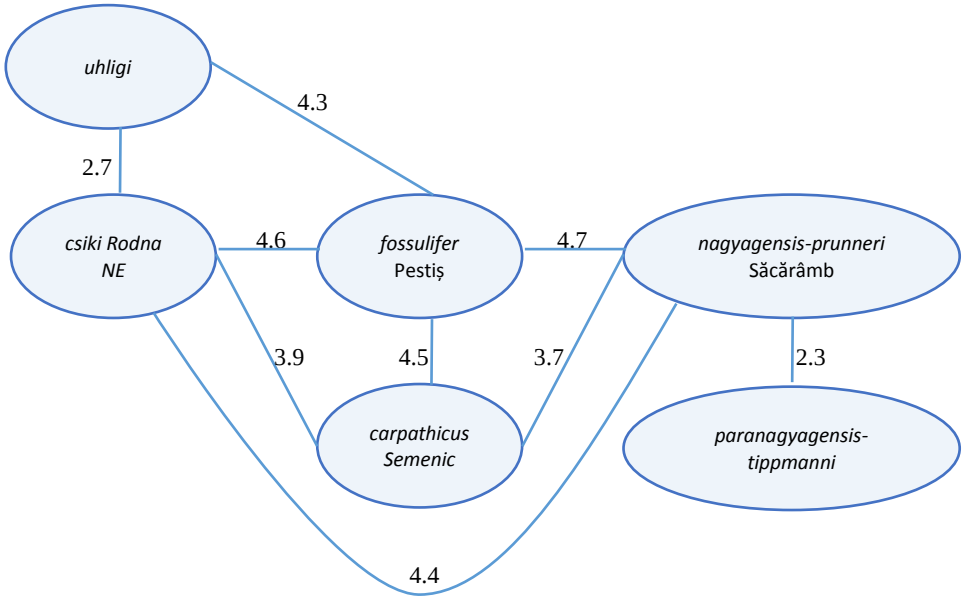

Fig. 1. Genetic distance in percentage

In Sacaramb (Metaliferi Mountains), homeland of *nagyagensis* are found in a mixture, in the same stations *nagyagensis* and *paranagyagensis* (26%), morphologically indistinguishable. *Uhligi* and *csiki* Rodnaare quite close (genetic distance 2.7%).

Apart from *uhligi* and *paranagyagensis*, the genetic distances are between 3.7 and 4.7%.

Results for territorial sampling. Global analysis

After concatenation COI I/cyt b study, the 66 Romanian populations are classified in genetically homogeneous groups, attached or not to the previous types subspecies and presented in Appendix 1 (12 groups with intrapopulation variability analyses).

Taking into account the genetic divergence threshold limit of 2.3% beyond which another genetic group appears, we note (Figure 2):

- Besides the 6 subsp. previously identified: *csiki Rodna/uhligi/fossulifer/nagyagensis/carpathicus/(paranagyagensis)*
- 5 additional groups: *csiki NE* (Telciu type)/*csiki NW* (Lunca la Tisa type) et *uhligi obsoletus Lotru*
- *obsoletus Cozia* (Cozia, Stanisoara Monastery type)
- *obsoletus Parang-Poiana Rusca*

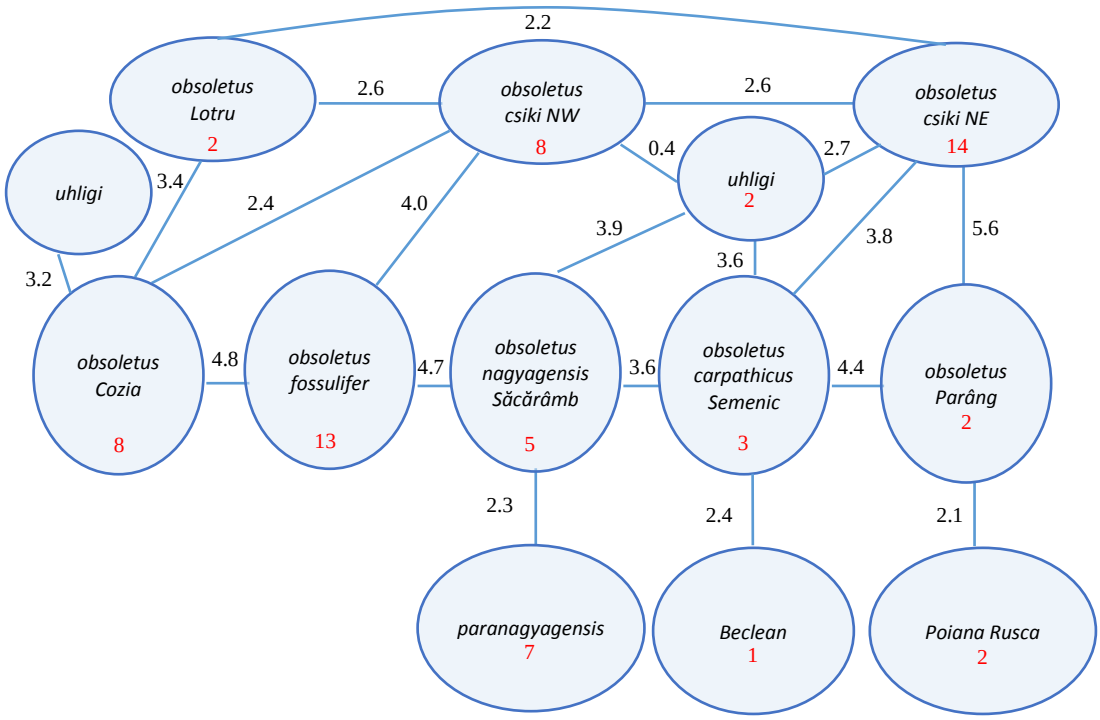

Fig. 2. Main differentiated groups (genetic distance in percentage and in red the number of analysed exemplars)

The number of specific bases to the main identified groups.
The number of specific bases compared to 1100 detected.

Table 3. The specific base numbers of some groups

Group	Number of specific bases
Parâng	17 (11 communes with Poiana Rusca)
fossulifer	11
nagyagensis	9
Cozia	6
Lotru carpathicus	4
csiki NE/Rodna	3
csiki NW/uhligi	1

Results by reference to populations close to the species of Sturm

According to Birthler [22], the Sturm exemplar describing *obsoletus* comes from Moravia Silesia (CZ). The species is still present in this region and in South Moravia, with rare specimens (Kutany National Reserve Spizer [23]).

We have a provenance from Moravian Silesia (place Stramberk leg V. Slovak) of *obsoletus obsoletus* which we consider to be close to the type. We have added two provenances from the Slovak Republic where the species is more common: *E. obsoletus obsoletus* from Remetske Hamre (leg. V. Slovak), Eastern region; *E. obsoletus aurocupreus* Reitter from Stara Huta (leg. V. Slovak) Central region.

The Romanian origins for sculptural morphology belongs:

- either to csiki (Malasz 1900) with absent or attenuated intervals on the disk and superficial foveas (*csiki NE, csiki NW, uhligi* Holdhaus 1910).
- either to other ssp: from the Lotru Mountains attached to *carpathicus* and *obsoletus Cozia* with the type sculpture.

The figure 3 shows the links between these origins:

**obsoletus* Moravia and *obsoletus* Slovakia are close (genetic divergence 0.7%)

**obsoletus Rodna*, type Cisa and *obsoletus csiki NE* (Telciu type are very close (genetic distance 0.01%) and get closer to Moravian obsoletus from which they could derive, having a maternal origin, no doubt close. *Obsoletus csiki* occupies in Romania a vast northern territory.

The whole ensemble *obsoletus CK-SK/obsoletus Rodna/obsoletus csiki NE* is sufficiently homogeneous to consider it as the typical group of the species.

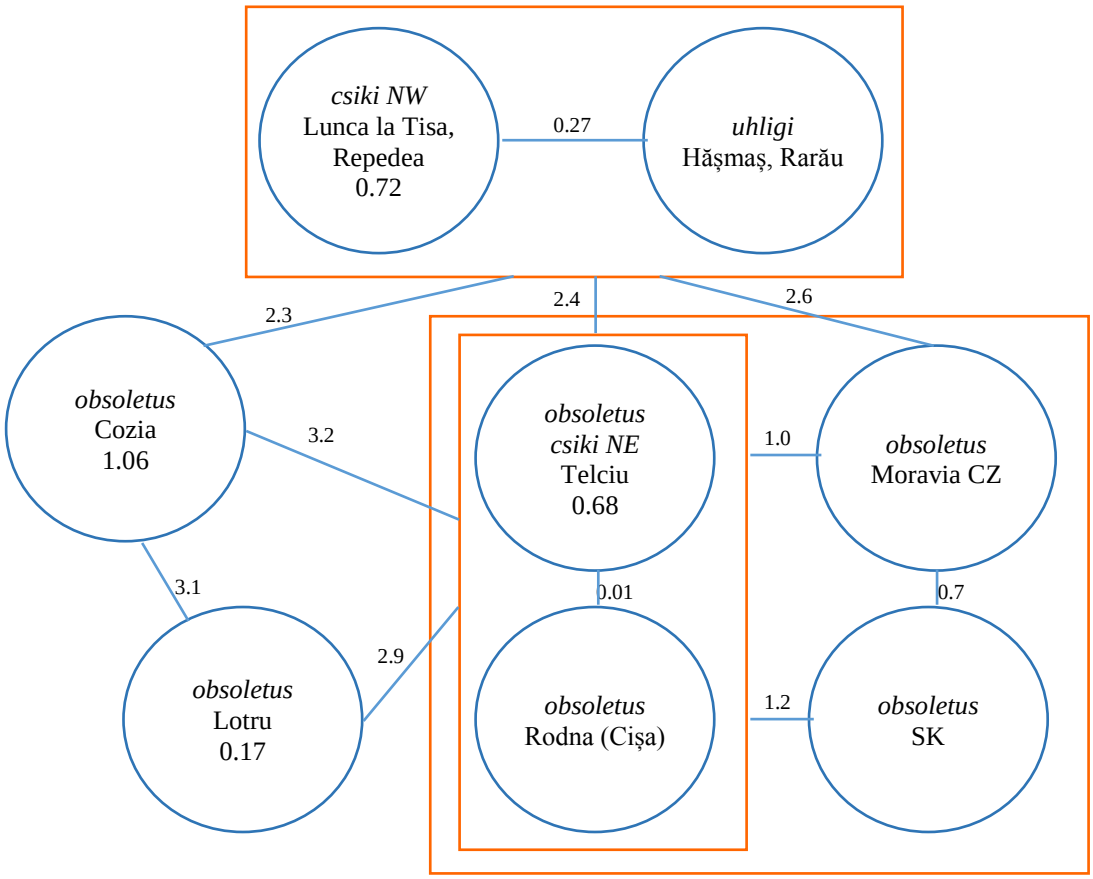

Fig. 3. Genetic distance in percentage

**uhligi*, a species of altitude (Rarau Mountains, Hasmas) is very close to *csiki NW* (Lunca la Tisa type, genetic distance 0.27); the latter form was not very far (difference 26 to 28 bp) from *obsoletus Cozia* and *obsoletus Lotru* with divergence percentages from 2.3 to 2.6%.

The table 4 lists the specific base pairs in the *csiki NE/csiki NW-uhligi/obsoletus NE-Rodna/obsoletus Cozia/obsoletus Lotru*group, among 42 of them. Despite genetic differences between these provenances close to the chosen differentiation threshold (2.2 to 2.3%), *obsoletus* Lotru and *obsoletus* Cozia have an original genetic structure and a structural morphology distinct of csiki.

These subspecies, well characterized on the genetic point of view and the well-established localization differ from each other and from other groups by high genetic divergence rates (3.5 to 4.9% Fig.2).

Table 4. Number of specific bases

Group	Number of specific bases
*obsoletus csiki NW/uhl*igi	*6*
*obsoletus csiki NE/obsoletus*Rodna	*11*
obsoletus Lotru	*11*
obsoletus Cozia	*14*

obsoletus Parang/Poiana Rusca (Fig. 4, Fig. 5).

The origin of the Parang Mountains differs strongly from all other identified origins, with a genetic distance between 4.4 and 6.0%. Poiana Rusca (Gosta station) gets closer with 20 bp of difference, 2 of which are in substitution.

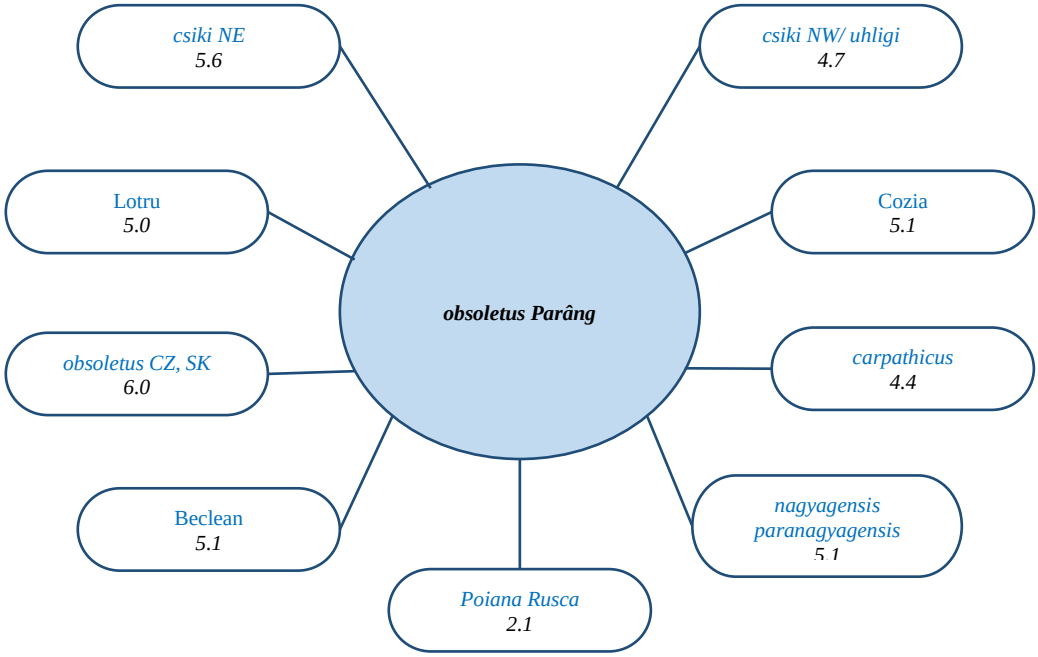

Fig. 4. Genetic distance from *obsoletus Parâng*

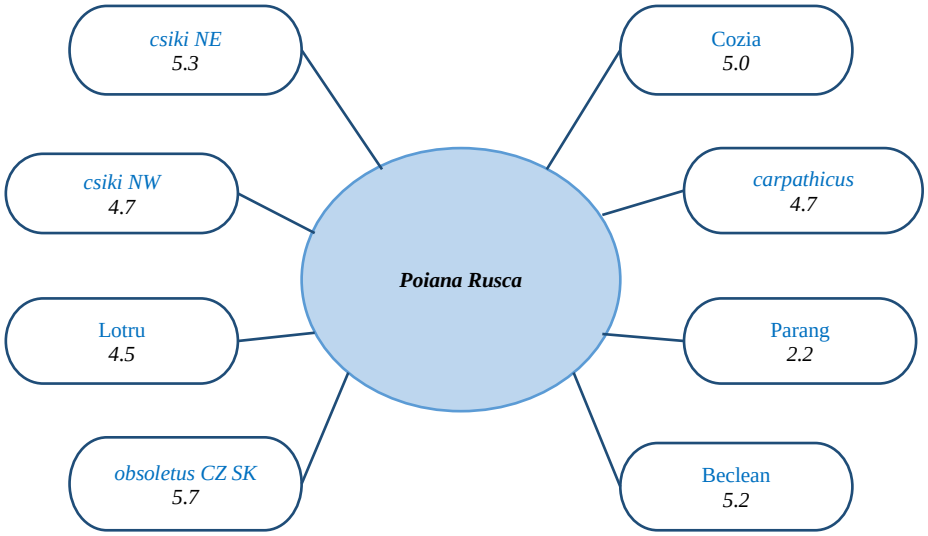

Fig. 5. Genetic distance from *obsoletus Poiana Rusca*

The form from the Parang Mountains is genetically the most diverse of all identified groups. It deserves to be retained as a new subspecies.

Brief description of obsoletus Parang (new subspecies)

Obsoletus from Parâng is a subspecies of small size males L/l=20.7/8.5 mm (extreme values L/l=19.0-22.5/5.9-6.6 mm), females L/l=21.5/9.1 mm (extreme values L/l=20.5-24.0/6.0-7.0 mm).

These dimensions bring it closer to *C. (Eucarabus) obsoletus carpathicus euchromus* Palliardi 1825, from the summit populations of the Semenic Mountains (males L/l=21.2/8.3, females L/l=22.1/8.7 mm., Barloy [24]).

The colour is characterized by a dominant two-colour: pronotum brown red/elytra yellowish green (38% population) or brown red, brown more or less dark 44% and only 18% of melanizing individuals (dark purple withgreen edges of the pronotum and elytra) more abundant in the higher altitudes. Pronotum with short and rounded posterior angles, its dimension ratio being that of other subspecies.

The sculpture is characterized by salient intervals, the secondary and tertiary having the same width and the primaryinterrupted by foveoles well-marked, in segments often quite short.

After the third primary interval, there is one whole interval (47% of males, 87% of females) then are granules often arranged in lines, 12% of the population (female insects) having two more whole intervals.

Species of alpine meadow (1000-2100 m) more abundant in grassy area or under the cover of *Pinusmugo*. In the rocky zone where it is rarer, it coexists with *C. (Oreocarabus) linnei* Panzer 1810 and especially *C. (Oreocarabus) silvestristransylvanicus* Dejean 1826.

As always in the mountains, the starting date of adult activity depends on the melt of snows (usually late May-early June). The main activity period is quite short but intense; displacement in the sun in the appropriate biotopes.

It is not certain if this form is only present in the Parang Mountains population or is extended at high altitude in nearby mountains.

Conclusions

C. (Eucarabus) obsoletus is a species for which subspecific variability is difficult to establish on the basis of the morphological criteria but the use of mitochondrial markers (COI I/cyt b) easily and unambiguously differentiates the subspecies.

For the described subspecies, this technique confirms with high rates of genetic divergence (3.6 to 4.7%), the originality of *csiki* Rodna and *fossulifer*, well distinguished from *nagyagensis, carpathicus* and *uhligi* but does not recognize *prunneri*, assimilated to *nagyagensis*, or*tippmani* not distinct from *paranagyagensis*, neighbour of the *nagyagensis*.

The study:

- reveals close links with the presumed type from Moravia for Mount Rodnei provenances and a series of localities from the NE of Romania
- shows the proximity of a group genetically related to *csiki NE-NW* but morphologically different, the most original being the Cozia origin.
- detects a new form of the Parang Mountains (may be Poiana Rusca) with strong genetic divergence
- indicates a very high infraspecific genetic variability (genetic divergence 3.0 to 5.6 %)

The strongest genetic differentiation has occurred in the Romanian Western Carpathians (especially Transylvanian) and may be locally in the southern Carpathians.

Annexe 1. Groups of *C. (Eucarabus) obsoletus*

Group *csiki* Rodna and *csikiNE* Genetic distance 0.68%			
	Station	**Latitude** **longitude**	**Altitude**
1	Borşa	47°39 N 24°38 E	1000 m
2	Cisa (Rodna Mountains)	47°32 N 24°49 E	1800 m
3	Romuli	47°32 N 24°26 E	660 m
4	Telciu	47°28 N 24°53 E	390 m
5	Telcişor	47°28 N 24°26 E	500 m
6	Bichigiu	47°25 N 24°21 E	430 m
7	Corongiş	47°30 N 24°48 E	1300 m

8	Colibița	47°10 N 24°50 E	670 m
9	Sărățel	47°02 N 24°24 E	460 m
10	Suplai	45°25 N 24°16 E	675 m
Group *csiki NW* Genetic distance 0.72%			
	Station	Latitude longitude	Altitude
11	Lunca la Tisa	47°56 N 24°01 E	350 m
12	Repedea	47°49 N 24°24 E	480 m
13	Ruscova	47°48 N 24°17 E	350 m
14	Bocicoel	47°43 N 24°19 E	550 m
15	Lac Bodi	47°40 N 23°46 E	780 m
16	Gutâi Pass	47°41 N 23°46 E	980 m
17	Băiuț	47°35 N 23°58 E	710 m
18	Mestecăniș Pass (VatraDornei)	47°28 N 25°21 E	1060 m
19	Răstolnița	46°28 N 24°59 E	630 m
20	Andreasa	46°57 N 25°03 E	645 m
Group *uhligi* Genetic distance 0.15%			
21	Hășmaș	46°42 N 25°48 E	1790 m
22	Rarău Mountains	47°27 N 25°33 E	1500 m
23	Vlăhița	46°19 N 25°33 E	800 m
Group *carpathicus* Genetic distance 1.02%			
24	Semenic	45°11 N 22°04 E	1380 m
25	Topenie Valley	45°03 N 22°34 E	540 m
26	Beclean	47°43 N 24°19 E	270 m
obsoletus "Lotru" Genetic distance 0.45%			
27	Voineasa	45°25 N 23°57 E	850 m
obsoletus "Cozia" Genetic distance 1.06%			
28	Sfânta Ana Lake	46°07 N 25°54 E	1110 m
29	Poiana Brașov	45°37 N 25°33 E	930 m
30	Cozia (Meteo Station)	45°19 N 24°20 E	1560 m
31	StânișoaraMonatery	45°18 N 24°20 E	760 m
32	Păltiniș	47°28 N 25°30 E	1070 m
33	Bogata Forest Pass	45°54 N 25°26 E	700 m
34	Sărățeni	46°33 N 25°00 E	670 m
35	Viperești	45°14 N 26°30 E	213 m
36	Cozia forest	45°17 N 24°19 E	425 m
Group *fossulifer* Genetic distance 1.15%			
37	Pestiș	47°06 N 22°24 E	300 m
38	Șinteu	47°07 N 22°26 E	677 m
39	Betfia	46°58 N 22°00 E	170 m
40	Băile Felix	46°59 N 21°58 E	150 m
41	Hidișelu de Jos	46°57 N 22°01 E	210 m
42	Hidișelu de Sus	46°56 N 22°05 E	310 m
43	Meseș	47°09 N 23°05 E	600 m
44	Firminiș	47°14 N 23°08 E	250 m
45	Răchițele	46°41 N 22°52 E	1000 m
46	Aleșd	47°03 N 22°23 E	410 m
47	SomeșuRece	46°41 N 23°20 E	530 m
48	Galbena Valley	46°33 N 22°39 E	700 m
49	Bucium Pass	46°13 N 23°00 E	710 m
50	Molod	46°46 N 22°11 E	220 m

51	MăguriRăcătău	46°37 N 23°10 E	850 m
52	Bihor (Boga)	46°35 N 22°39 E	639 m
Group *nagyagensis* Genetic distance 0.56%			
53	Săcărâmb	45°58 N 23°01 E	600 m
54	Scărișoara	46°28 N 22°49 E	730 m
55	Băișoara	46°34 N 23°23 E	820 m
56	Remetea (BH)	46°44 N 22°21 E	180 m
57	Poiana Aiudului	46°21 N 23°33 E	430 m
Group *prunneri* Genetic distance 0.56%			
58	Bucium village	46°15 N 23°10 E	734 m
59	Buceș-Vulcan Pass	46°13 N 23°00 E	710 m
60	Detunata Goală	46°16 N 23°11 E	1160 m
Group *paranagyagensis* Genetic distance 0.62%			
61	Zlatna	46°06 N 23°11 E	500 m
62	Zam	46°00 N 22°26 E	200 m
63	Pojoga	45°59 N 22°22 E	140 m
64	Bârzava-Nadăș	46°08 N 21°59 E	165 m
Group *"Parang"* Genetic distance 0.45%			
65	Gosta, Nădrag	45°39 N 22°13 E	600 m
66	Urdele Pass (Parâng)	45°22 N 23°39 E	1830 m 2145m
Czech and Slovak Republic			
67	Moravia CZ (Stamberk)	49°35 N 18°17 E	415 m
68	Slovak Republic SK (Remetska Hamre)	48°50 N 22°11 E	288 m
69	Slovak Republic SK (Stara Huta)	48°28 N 19°20 E	772 m

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