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Comparison of molecular and morphological systematics of *Carabus* species (Coleoptera: Carabidae) with special emphasis on species from Dinaric karst

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Abstract

Background and Purpose*: Despite morphological and molecular data analysed so far, phylogenetic relationships of many lineages of genus* Carabus *Linnaeus 1758 (Carabini, Carabinae, Carabidae) present in Europe, have not been yet fully understood and molecular data have not been fully integrated with the morphological classifications. The aim of this research was to: (i) complement the phylogenetic relationships in the systematics of carabids within the genus* Carabus *with endemic species from Dinaric karst not included so far in molecular systematics research, and to (ii) examine the degree of matching of the molecular data with the existing morphological classifications based on the structure of frontal shields of larvae and the structure of the endophallus in adult specimens.*

Materials and Methods*: In this research, phylogenetic relationships between 31 species from genus* Carabus *were analysed. Analyses were based on the DNA sequences of mitochondrial gene for cytochrome c oxidase subunit I. For phylogenetic inference maximum likelihood and Bayesian analysis methods were used.*

Results: *The obtained results showed a greater concordance of molecular data with the classifications based on the structure of endophallus than with the classification based on structure of frontal shields of larvae. The results mainly corresponded to the phylograms in the previous studies confirming the taxonomic status of some species. For three species,* Carabus creutzeri *and* C. parreyssi *alpine-dinaric endemic species and* C. ulrichi*, all included for the first time in molecular analysis, results indicated taxonomic congruence of molecular data with the classification based on the structure of endophallus.*

Conclusions: *Systematic categories within the genus* Carabus *cannot be based only on the structure of the endophallus, but the results of molecular analysis should also be included. As the topology of several groups still remains uncertain, molecular phylogeny requires further investigations with a larger data set and additional molecular markers.*

Carabus *species from the western part of Balkan Peninsula, mainly endemics, have been under-represented in hitherto molecular systematic analyses. Further studies, including their distribution and ecology as well as studies including other Balkan's endemic species are required to explain speciation events. This study contributes to the Barcode of Life Initiative.*

INTRODUCTION

The genus *Carabus* Linnaeus 1758 belongs to the subfamily Carabinae, family Carabidae, suborder Adephaga and order Coleoptera. It is the most numerous genus within Carabidae. Genus *Carabus* comprises about 940 valid species classified into 91 subgenera *(1).* 132 species occur in Europe while thirty species have been recorded in Croatia *(2),* including 53 subspecies and 81 forms and aberrations *(2).* Almost all species occur in the Palaearctic region with a dozen species occurring in the Nearctic *(3).*

Carabus species represent the largest Carabids, with body size from around 12 to 50 mm long. They are mostly wingless, nocturnal predators of snails, earthworms and caterpillars in forest and open habitats *(4, 5, 6). Carabus* species as distinctly terricolous and mostly non-flying insects are very sensitive to habitat fragmentation and changing environmental variables *(7),* and are therefore, considered as good bioindicators *(8).*

Within genus *Carabus* several species are endangered and listed in the European list of endangered species (IUCN). Three of them: *Carabus (Platycarabus) creutzeri* Fabricius 1801, *Carabus (Chaetocarabus) intricatus* Linnaeus 1761 and *Carabus (Eucarabus) parreyssi* Palliardi 1825, included in this research, have been placed in a number of national Red lists (i.e. Croatian Red List).

Carabus (Platycarabus) creutzeri Fabricius 1801 is an endemic species of the Central and Eastern Prealps and Alps on the southern side. This is a typical mountain species, occurring in bushes and forests, mostly in the alpine zone (2000-2300 m a.s.l.: 9), but occasionally also at lower altitudes (200-300 m a.s.l. in few places in Veneto: 10). In North - Western Balkans *C. creutzeri* occurs from the low valley forests (200 m a.s.l.) to open habitats at high altitudes (2500 m a.s.l.: 11). Pavičević and Mesaroš *(11)* mention the form *humilis* Berneau as possibly endangered. According to Winkler *(12)* and Drovenik and Peks *(13) Carabus creutzeri humilis* is Croatia's endemic subspecies. *Carabus (Chaetocarabus) intricatus* Linnaeus 1761 is an endangered species listed in the European Red List of Threatened Species (IUCN). It occurs up to 1700 m a.s.l. and is a good indicator of maintenance of old-growth forests and soil rich in organic matter *(14, 15). Carabus (Eucarabus) parreyssi* Palliardi 1825 is an endemic to North-Western part of Balkan Peninsula. According to Pavičević and Mesaroš *(11)* all forms are endangered. Generally it occurs in very large, undisturbed forests *(10),* however according to Pavičević and Mesaroš *(11),* it is regarded as a species of subalpine habitats which occurs in open habitats in hills and mountains as well.

Despite numerous studies, phylogenetic relationships within the genus *Carabus* are still unresolved. The two most well-known classifications based on morphological characteristics are the classification based on the structure of frontal shields of larvae and the classification based on the structure of endophallus in adults. The first one divided genus *Carabus* into three large groups - Archeocarabi, Metacarabi and Neocarabi *(16).* The classification based on the structure of endophallus, first suggested by Ishikawa (1978), divided the genus into eight groups: Spinulati, Digitulati, Lipastrimorphi, Archicarabomorphi, Tachypogenici, Arcifera and Neocarabi *(17).* The classification based on the structure of endophallus is more widely used nowadays, than classification based on the structure of larval shields *(1, 17).*

Phylogeny based on ND5 gene by Sota and Ishikava *(18)* gave the first picture of the relationships among *Carabus* subgenera but failed to resolve the fundamental relationships within the genus. These results did not corroborate classifications based on morphological characters (19 – 23). Deuve *et al. (24)* produced the first phylogeny of the genus *Carabus* based on both mitochondrial and nuclear markers in research which included *Carabus* species from the Iberian and Balkan Peninsula while Andujar *et al. (3)* conducted calibration analyses for the genus *Carabus* combining five mitochondrial and four nuclear DNA fragments to find out the rates of molecular evolution for this genus.

Genus *Carabus* is known as a hyper-diverse genus *(1).* Diversity is specially noticed in areas labelled as refuge. Of the different refuge areas, the Balkan Peninsula is considered as the most taxon-rich *(25, 26),* with Dinaric karst, referred as the greatest natural treasure of the Balkan Peninsula. Sket *(27)* pointed out a rich geological history of Dinaric karst and high diversity of flora and fauna of that specific area. Therefore, Balkan Peninsula is placed among 25 World's biodiversity hotspots *(28).*

Here, in this research, we used for the first time mitochondrial sequences from two endemic species, *Carabus (Platycarabus) creutzeri and Carabus (Eucarabus) parreyssi,* and another *Eucarabus* species *Carabus ulrichi* in reconstruction of the *Carabus* phylogenetic tree.

The aim of this research was to complement the phylogenetic relationships in the systematics of Carabids within the genus *Carabus* Linnaeus 1758 (Carabini, Carabinae, Carabidae) with some endemic species from Dinaric karst and to check the matching of phylogenetic trees with existing morphological classifications based on the structure of frontal shields of larvae and the structure of endophallus in adult specimens.

MATERIALS AND METHODS

Taxonomic sampling

Our phylogenetic analysis included 31 species of genus *Carabus*, belonging to 18 different subgenera. The specimens used in this analysis are listed in Table 1. All the main subgroups (Archicarabomorphi, Arcifera, Digitulati, Li-

TABLE 1

List of *Carabus* species included in this study, with division according to endophallus (17) and larval morphology (10, 41 – 43), and outgroup species included in this study with accession numbers from the GenBank and localities of individual species.

No.	Species/Sequence	*Carabus* division (endophallus morphology)	*Carabus* division (larval morphology)	Locality/ GenBank	GenBank Accession number
1	*Archicarabus nemoralis*1	ARCHICARABOMORPHI	ARCHEOCARABI	Croatia: Medvednica	KP067550
2	*Archicarabus nemoralis*2	ARCHICARABOMORPHI	ARCHEOCARABI	Croatia: Medvednica	KP067554
3	*Archicarabus nemoralis*3	ARCHICARABOMORPHI	ARCHEOCARABI	Croatia: Medvednica	KP067548
4	*Archicarabus nemoralis*4	ARCHICARABOMORPHI	ARCHEOCARABI	Croatia: Medvednica	KP067547
5	*Archicarabus nemoralis*5	ARCHICARABOMORPHI	ARCHEOCARABI	Croatia: Medvednica	KP067547
6	*Archicarabus nemoralis*6	ARCHICARABOMORPHI	ARCHEOCARABI	Croatia: Medvednica	KP067549
7	*Chaetocarabus intricatus*1	ARCIFERA	NEOCARABI	Croatia: Medvednica	KP067569
8	*Chaetocarabus intricatus*2	ARCIFERA	NEOCARABI	Croatia: Medvednica	KP067569
9	*Chaetocarabus intricatus*3	ARCIFERA	NEOCARABI	Croatia: Medvednica	KP067570
10	*Chaetocarabus intricatus*4	ARCIFERA	NEOCARABI	Croatia: Medvednica	KP067572
11	*Chaetocarabus intricatus*5	ARCIFERA	NEOCARABI	Montenegro: Durmitor	KP067571
12	*Platycarabus creutzeri*1	ARCIFERA	NEOCARABI	Croatia: Velebit	KP067564
13	*Platycarabus creutzeri*2	ARCIFERA	NEOCARABI	Croatia: Velebit	KP067563
14	*Platycarabus irregularis*	ARCIFERA	NEOCARABI	GenBank	JQ689887
15	Carabus arvensis	DIGITULATI	ARCHEOCARABI	GenBank	JQ646568
16	Carabus deyrollei	DIGITULATI	no data	GenBank	JQ646588
17	*Eucarabus parreyssi*1	DIGITULATI	no data	Croatia: Velebeit	KP067568
18	*Eucarabus parreyssi*2	DIGITULATI	no data	Croatia: Poštak	KP067567
19	*Eucarabus catenulatus*1	DIGITULATI	no data	Croatia: Gorski Kotar	KP067558
20	*Eucarabus catenulatus*2	DIGITULATI	no data	Croatia: Gorski Kotar	KP067557
21	*Eucarabus catenulatu*3s	DIGITULATI	no data	Croatia: Gorski Kotar	KP067559
22	*Eucarabus sternbergi*	DIGITULATI	no data	GenBank	HM180573
23	*Eucarabus ulrichi*1	DIGITULATI	ARCHEOCARABI	Croatia: Medvednica	KP067556
24	*Eucarabus ulrichi*2	DIGITULATI	ARCHEOCARABI	Croatia: Medvednica	KP067555
25	*Morphocarabus monilis*1	LIPASTRIMORPHI	ARCHEOCARABI	GenBank	GU347147
26	*Morphocarabus monilis*2	LIPASTRIMORPHI	ARCHEOCARABI	GenBank	GU347148
27	*Eurycarabus famini*1	METACARABI	METACARABI	GenBank	JQ689884
28	*Eurycarabus famini*2	METACARABI	METACARABI	GenBank	JQ689878
29	*Mesocarabus lusitanicus*	METACARABI	no data	GenBank	JQ689901
30	Mesocarabus macrocephalus	METACARABI	no data	GenBank	JQ689879
31	*Nesaeocarabus abbreviatus*1	METACARABI	METACARABI	GenBank	JQ689894
32	*Nesaeocarabus abbreviatus*2	METACARABI	METACARABI	GenBank	JQ689874
33	*Oreocarabus hortensis*1	METACARABI	METACARABI	Croatia: Dalmatia	KP067566
34	*Oreocarabus hortensis*2	METACARABI	METACARABI	Croatia: Gorski Kotar	KP067565
35	Orinocarabus baudii	METACARABI	no data	GenBank	JQ646601
36	Orinocarabus fairmairei	METACARABI	no data	GenBank	JQ646595
37	*Tomocarabus convexus*1	METACARABI	METACARABI	Croatia: Medvednica	KP067546
38	*Tomocarabus convexus*2	METACARABI	METACARABI	Croatia: Medvednica	KP067552
39	*Tomocarabus convexus*3	METACARABI	METACARABI	Croatia: Medvednica	KP067553
40	Chrysocarabus auronitens	NEOCARABI	NEOCARABI	GenBank	GU347140
41	*Chrysocarabus rutilans*	NEOCARABI	NEOCARABI	GenBank	JQ689891
42	Macrothorax morbillosus	NEOCARABI	NEOCARABI	GenBank	JQ689883
43	Macrothorax rugosus	NEOCARABI	NEOCARABI	GenBank	JQ689882
44	Megodontus caelatus	NEOCARABI	NEOCARABI	Montenegro: Durmitor	KP067573
45	*Megodontus violaceus*1	NEOCARABI	NEOCARABI	Croatia: Medvednica	KP067561
46	*Megodontus violaceus*2	NEOCARABI	NEOCARABI	Croatia: Medvednica	KP067562
47	*Procrustes coriaceus*1	NEOCARABI	NEOCARABI	Croatia: Medvednica	KP067574
48	*Procrustes coriaceus*2	NEOCARABI	NEOCARABI	Croatia: Medvednica	KP067574
49	*Apotomopterus kouanping*	SPINULATI	no data	GenBank	JQ646606
50	*Apotomopterus skyaphilus*	SPINULATI	no data	GenBank	JQ646604
51	Tachypus auratus	TACHYPOGENICI	ARCHEOCARABI	GenBank	JQ646600
52	Tachypus cancellatus	TACHYPOGENICI	ARCHEOCARABI	Croatia: Dalmatia	KP067551
53	Tachypus cristofori	TACHYPOGENICI	no data	GenBank	JQ646597
54	Cychrus caraboides	outgroup		GenBank	AB109838
55	Cychrus attenuatus	outgroup		Croatia: Medvednica	KP067560

pastrimorphi, Metacarabi, Neocarabi, Spinulati and Tachypogenici) as defined by the endophallic characters of male genitalia were represented *(17)*. Also, two species of *Cychrus,* which served as an outgroup to root the phylogenetic trees were included. 32 DNA sequences from 12 species of *Carabus* and one *Cychrus* species were obtained by DNA extraction and sequencing from specimens collected in the field, while the rest 23 sequences were retrieved from the NCBI GenBank database (Table 1).

Ground beetles were sampled in the area of Mt. Medvednica (Croatia), from May to October 2007, Mts. Velebit (Croatia) and Durmitor (Monte Negro) in 2009, Mt. Poštak and Krka riverside in Dalmatia (Croatia) in 2010 and Gorski Kotar area (Croatia) in 2010 and 2013, (Table 1). Immediately after collecting, samples were stored in 96% EtOH until the beginning of laboratory processing.

DNA extraction, amplification and sequencing

DNA was extracted using the Qiagen „DNeasy Tissue Kit", following standard protocols. The reaction mixture for PCR was prepared according to the "HotMasterMix (2.5X)" (Eppendorf) reagent kit manufacturer's instructions. Amplification of the Cytochrome Oxidase Subunit I (COI) gene fragments was accomplished by using LCO-1490 (5' – GGTCAACAAATCATAAAGATATTGG – 3') and HCO-2198 (5' – TAAACTTCAGGGTGACCAAAAAATCA – 3') primers (29). Polymerase chain reaction began with a two minute initialization step at 94 °C, followed by 35 cycles of denaturation for 2 minutes at 94 °C, primer annealing for 45 seconds at 45 °C and fragment elongation for 1 minute at 65 °C. Each program ended with the final reaction of DNA synthesis lasting 7 minutes at 65 °C. PCR products were purified using Roche „High Pure PCR Product Purification Kit", following the manufacturer's instructions, and sequenced in one direction in Macrogen Inc. company (South Korea) using the LCO-1490 primer (29). Sequences are deposited in GenBank under accession numbers listed in Table 1.

Phylogenetic analyses

Our sample consisted of 55 COI sequences (53 *Carabus* + 2 outgroups), 560 bp long. The sequences were aligned using default settings in ClustalW (implemented in MEGA 5 *(29)*). The most appropriate evolutionary model for our data set was identified as TPM1uf+I+G, using the corrected Akaike information criterion implemented in jModelTest 2.1.1 *(30)*.

Phylogenetic trees were reconstructed using maximum likelihood and Bayesian methods. Maximum likelihood (ML) analysis was performed using PhyML 3.0 *(31)*. We used a custom TPM1uf model with fixed gamma shape parameter (0.531) and proportion of invariable sites (0.352), both chosen according to the results of the jModelTest analysis. The number of substitution rate categories was set to 4. Tree topology search operations were set to best of NNIs and SPRs, starting from 5 random trees. To estimate the node reliability, we used approximate likelihood ratio test with SH-like supports.

Bayesian analysis (BA) was conducted using MrBayes 3.1.2 *(32)*. We used the same model parameters (nst = 6, shapepr = 0.531, pinvarpr = 0.352) as in ML analysis. To improve mixing of the cold chain and prevent it from becoming trapped in local optima, we used Metropolis-coupled Markov chain Monte Carlo (MCMC) method, with each run including a cold chain and three incrementally heated chains (8 chains in total for two parallel runs). The heating parameter was set to 0.2. We ran the analysis for 1.000.000 generations with all the parameters and trees sampled every 100 generations. Convergence between the two runs was monitored in Mr. Bayes through the standard deviation of split frequencies, and runs were continued until this value dropped to less than 0.01. Then, the convergence of each run towards stationarity was monitored with Tracer v. 1.4 *(33)* using likelihood values as well as all other parameters estimated. Stationarity was reached after 250000 of generations. Hence, 2500 trees were discarded as burn-in.

After the summarizing consensus tree was assigned, the posterior probabilities were obtained accordingly.

RESULTS

The phylogenetic analyses based on two different methods of phylogenetic reconstruction (Maximum likelihood and Bayesian analysis) resulted in similar topologies (Figures 1 and 2).

Half of the eight subgroups (Lipastrimorphi, Spinulati, Archicarabomorphi and Digitulati) based on endophallic characters of the male genitalia were recovered as monophyletic in both trees. Tachypogenici were monophyletic only in the ML tree, with moderate support. The monophyly of Arcifera, Metacarabi and Neocarabi was not supported by our data. The clade comprising Digitulati + Archicarabomorphi + Neocarabi was strongly supported in the ML tree (0.92 SH), while it was not supported in the BA tree. Both trees suggested a close relationship between Spinulati and Tachypogenici (0.92 SH and 0.96 PP). Lipastrimorphi were not clustered within Carabogenici (Digitulati + Archicarabomorphi + Lipastrimorphi, 19) as was recorded in previous studies *(18, 24)*. In the ML tree Lipastrimorphi appeared as a sister group to the clade comprising subgenera *Oreocarabus*, *Nesaeocarabus* and *Eurycarabus* (Metacarabi), while in BA tree their relationship to other crown group members was unresolved.

All represented subgenera were monophyletic in both trees except for *Megodontus, Machrothorax* (only in BA tree) and *Tachypus* (moderate support in ML tree). Mono-

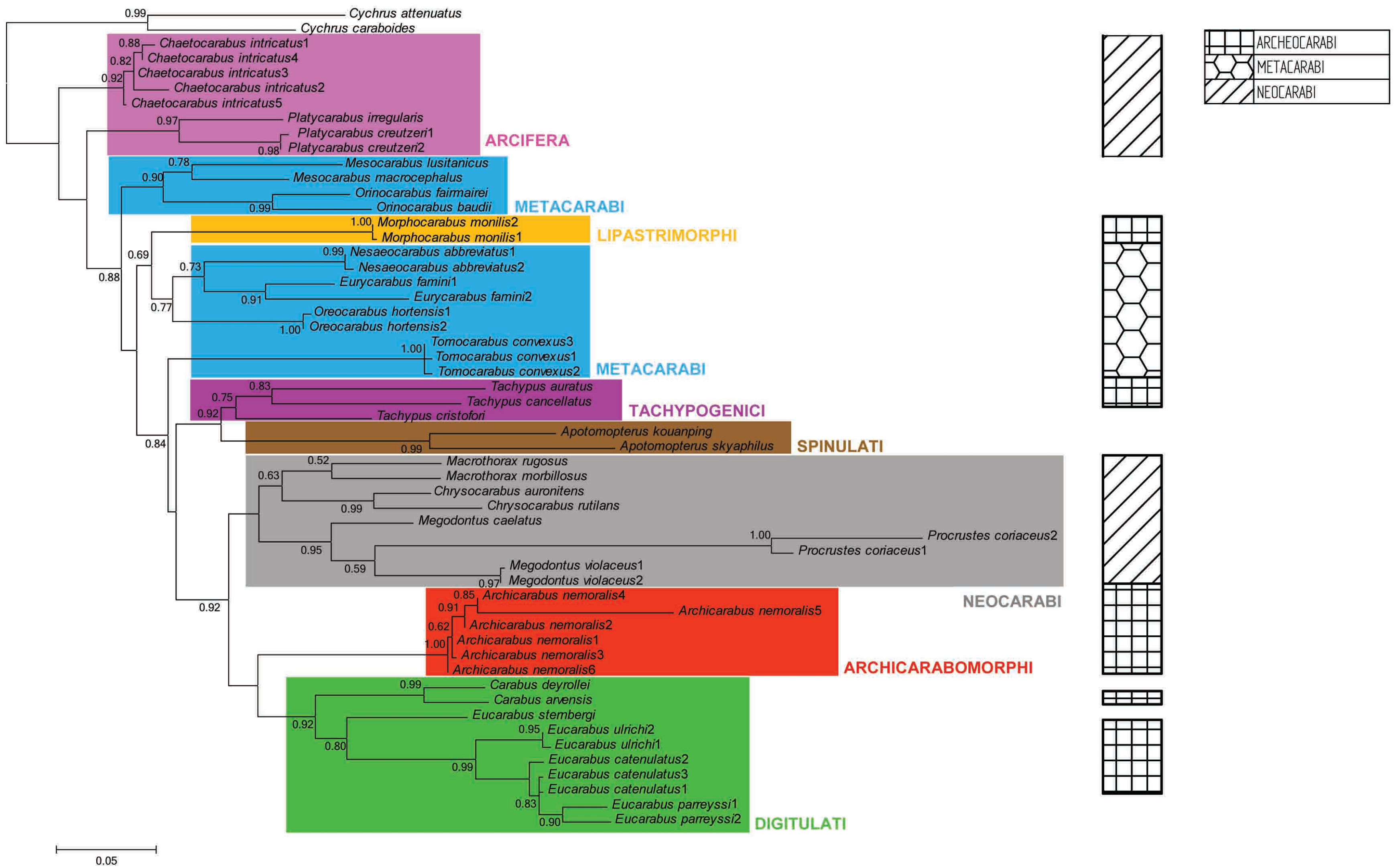

Figure 1. *The rooted Maximum Likelihood phylogram of 31 Carabus species and two outgroup Cychrus species under TPM1uf+I+G model of nucleotide substitution. Colours denote grouping based on the structure of endophallus. Different subgroups (Spinulati, Digitulati, Lipastrimorphi, Archicarabomorphi, Tachypogenici, Arcifera and Neocarabi) are covered by coloured rectangles, and the name of each subgroup is written using the same colour beside each rectangle. Groupings according to larval morphology (Archeocarabi, Metacarabi and Neocarabi) are shown in rectangles filled with lines and shown in the column beside the tree together with the explanatory legend. Numbers at nodes represent aLRT SH-like supports (only values ≥ 0.5 are shown).*

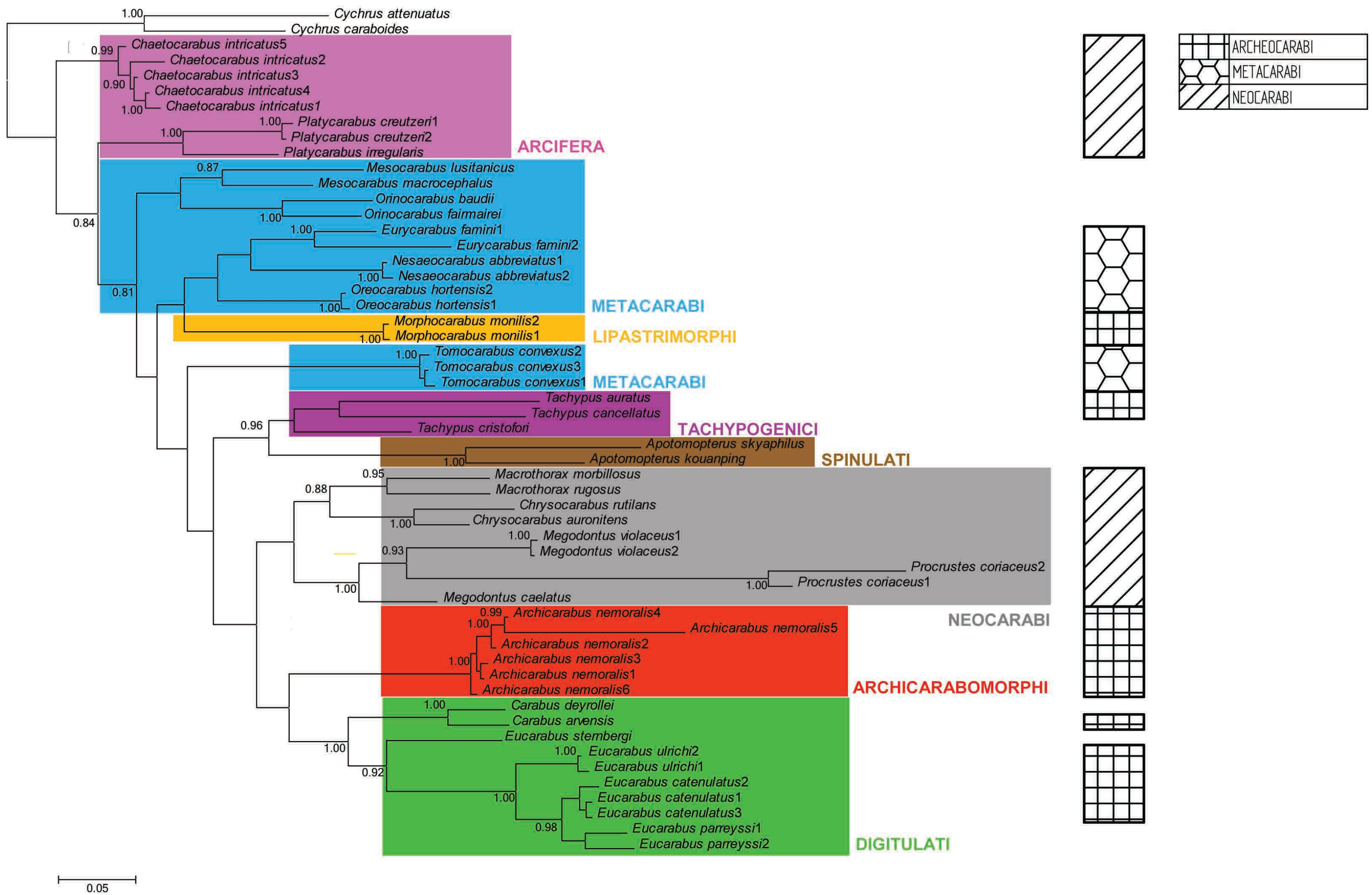

Figure 2. *The rooted Bayesian phylogram of 31 Carabus species and two outgroup Cychrus species using Metropolis-coupled Markov chain Monte Carlo (MCMC) method under TPM1uf+I+G model of nucleotide substitution. Different subgroups (Spinulati, Digitulati, Lipastrimorphi, Archicarabomorphi, Tachypogenici, Arcifera and Neocarabi) are covered by coloured rectangles, and the name of each subgroup is written using the same colour beside each rectangle. Groupings according to larval morphology (Archeocarabi, Metacarabi and Neocarabi) are shown in rectangles filled with lines and shown in the column beside the tree together with the explanatory legend. Numbers at nodes represent Bayesian posterior probabilities (only values ≥ 0.7 are shown).*

phyly of *Mesocarabus* was only moderately supported. On the ML tree subgenus *Orinocarabus* was sister to subgenus *Mesocarabus*, while subgenus *Eurycarabus* was sister to subgenus *Nesaeocarabus*. The same results were corroborated in previous research *(24).* Subgenus *Megodontus* was paraphyletic in both trees. *Megodontus caelatus* was sister to the clade comprising *Megodontus violaceus* + *Procrustes coriaceus*. Both trees supported the monophyly of the crown group excluding the two clades (*Chaetocarabus* and *Platycarabus*) traditionaly classified as Arcifera. The BA tree moderatly supported the sister status of *Platycarabus* relative to the crown group species, but this relationship was not supported in the ML tree.

Subgenus *Carabus* was sister to subgenus *Eucarabus* in both trees with good support. *Eucarabus ulrichi* and a clade comprising *Eucarabus parreyssi* and *Eucarabus catenulatus* were sister groups and formed a monophyletic group with other species belonging to the subgenera *Carabus* and *Eucarabus* (Digitulati).

DISCUSSION

According to Deuve *(1, 17),* the classification based on the structure of endophallus is nowadays more widely used than classification based on the structure of larval shields. Our results showed a greater concordance of molecular data with the former system than with the later one, justifying its increasing use. This is also supported by the fact that rapid and divergent evolution of male genitalia is one of the most widespread patterns of animal evolution *(34).*

This classification includes eight subgroups (Archicarabomorphi, Arcifera, Digitulati, Lipastrimorphi, Metacarabi, Neocarabi, Spinulati and Tachypogenici) and all of them were represented in this research by at least one species.

Our results largely corresponded to the phylograms obtained from the previous studies *(3, 18, 24). Chaetocarabus* and *Platycarabus* species (classified as Arcifera) were located at the base of the tree. In previous research subgroup Arcifera was found as a sister group to the remaining *Carabus* species *(18)* whereas in our study the position of Arcifera was unresolved probably due to a small number of species included.

In both trees the species *Megodontus caelatus* was sister to the clade comprising *Megodontus violaceus* + *Procrustes coriaceus*. The same result was obtained by Deuve et al. for mtDNA (24). When nuclear DNA was analysed, subgenus *Megodontus* was found monophyletic *(18, 24).* According to Sota and Vogler *(35)* the analysis of the nuclear genes provided data more compatible with the morphological classification than those made using mitochondrial DNA. However, mitochondrial genes have many advantages in molecular analysis and therefore are often used in constructing phylogenetic trees.

Digitulati, Archicarabomorphi and Lipastrimorphi grouped together in Carabogenici division *(19)* were not supported as monophyletic neither by Sota and Ishikawa *(18)* nor by our own study, but were not rejected by Deuve *et al. (24).*

However, our results must be considered with caution because two of the eight subgroups are represented by only one species (Archicarabomorphi and Lipastrimorphi), while additional two were represented by only one subgenus (Spinulati and Tachypogenici).

Within Digitulati *C. deyrollei* and *C. arvensis* were grouped together. The first one is a mountain species; endemic to Iberian Peninsula and the second one is a Palearctic species distributed thorough Northern and Central Europe and Siberia, all the way to Sakhlin, with no presence in Iberian Peninsula *(36).* In Croatia *C. arvensis* is present in Slavonia, the North-Eastern part of the country *(2).*

Eucarabus ulrichi and clade comprising *Eucarabus catenulatus* and *Eucarabus parreyssi,* the species of special interest for this research, were sister groups and monophyletic with other species belonging to the subgenera *Carabus* and *Eucarabus*, justifying their placement within the Digitulati subgroup. These two subgenera were also obtained as sister groups in previous researches *(18, 37).*

Eucarabus ulrichi inhabits deciduous forests and open habitats, from lowlands to the hills and mountains up to 500 m a.s.l. on warmer exposures in Central and South – East Europe *(38),* and in Croatia it comes in the North and North-Eastern part of the country *(39). Eucarabus catenulatus* and *Eucarabus parreyssi* have narrow distribution. *Eucarabus catenulatus* has Alpine-Dinaric distribution (South Switzerland, Central-North and North-Eastern Alps in Italy, Slovenia, Western Croatia and Bosnia and Herzegovina) *(38). Eucarabus parreyssi* is a species endemic to Dinaric Alps and distributed in South Croatia, North-West and South Bosnia and Herzegovina to the Northern part of Montenegro. These species are considered vicariant *(36),* geographically and ecologically, and thus have undergone genotypic and phenotypic divergence. Although, their adults morphologically resemble each other, males can be separated by the shape of their *edeagus*. According to the genetic data presented in this preliminary study, these species may still hybridise on the borders of their areals. The fact that these two closely related species were not clearly separated in neither of our trees attests to this possibility. In Croatia, *Eucarabus catenulatus* is present mainly in forests from lowland to mountain zones, more south-west exposed, on calcareous soil *(36),* while *Eucarabus parreyssi*, inhabits subalpine and alpine habitats (Šerić Jelaska, personal observations on Mt.Velebit).

Further insights into their distribution, ecological constrains and genetic differences between populations may give us more details on speciation events, as well as on some future prospects due to present environmental changes, such as climate change, that can impact the cold adapted high mountain species. The sequences for *C. ulrichi* and *C. parreyssi* have been used in this study for the first time to obtain the phylogenetic trees.

In congruence with previous studies *(3, 18, 24),* phylogenetic trees obtained in this study justify the more frequent use of the classification of the genus *Carabus* based on the structure of endophallus than of the other one, based on the frontal shields of larvae. Interpretation combining both, morphological and molecular data together is essential for obtaining the most precise phylogenetic reconstructions *(40). Carabus* species from the western parts of the Balkan Peninsula, mainly endemics, were so far underrepresented in molecular systematic analyses. As the topology of several groups still remains uncertain, further molecular phylogeny studies are required, with a larger number of species sampled, including other Balkan's endemic species, and additional molecular markers.

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