

Genetic diversity, phylogenetic position and morphometric analysis of *Astacus colchicus* (Decapoda, Astacidae): a new insight into Eastern European crayfish fauna

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Abstract

The phylogeny of European crayfish fauna, especially with respect to Eastern European species, is still far from being completely resolved. To fill this gap, we analyzed most of the European crayfish species focusing on the phylogenetic position of the endemic crayfish *Astacus colchicus*, inhabiting Georgia. Three mitochondrial and one nuclear marker were used to study evolutionary relationships among European crayfish species, resulting in the unique phylogenetic position of *A. colchicus* indicating independent species status to *A. astacus*. Phylogenetic analyses revealed a deep molecular divergence of *A. colchicus* in comparison to *A. astacus* (6.5–10.9% in mtDNA and 1.1% in nDNA) as well as to *Pontastacus leptodactylus* and *P. pachypus* (5.5–10.0% in mtDNA and 1.4–2.4% in nDNA). Absent ventral process on second male pleopod and abdominal somites II and III with pleura rounded lacking prominent spines clearly indicate taxonomic assignment to the genus *Astacus*; however, the species is distributed almost in the middle of Ponto-Caspian area typical by occurrence of the genus *Pontastacus*. Several morphological indices linked to head length, carapace, and total body length and width were found to demonstrate apparent differences between *A. colchicus* and *A. astacus*. Although this study provides a novel insight into European crayfish phylogeography, we also point out the gaps in comprehensive study of the *P. leptodactylus* species complex, which could reveal details about the potential species status of particular species and subspecies within this genus.

Key words: 12S rRNA, 16S rRNA, cytochrome c oxidase, histone H3, Ponto-Caspian crayfish

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INTRODUCTION

The Caucasus Mountains on the border between Asia and Europe, the Black and Caspian Seas, have played an important role in the formation of the current appearance of the Eurasian continent. In spite of a limited knowledge

of the region's biodiversity, the Caucasus are well known due to their high numbers of endemic species including plants, invertebrates, and vertebrate species (Mumladze *et al.* 2019). For instance, these include Caucasian rhododendron *Rhododendron caucasicum* Pallas, 1784; West Caucasian cave shrimp *Troglocharis kutaissiana* (Sadovskij, 1930); Buch's snail *Helix buchii* (Dubois de Montpéroux, 1840); Caucasian parsley frog *Pelodytes caucasicus* Boulenger, 1896; and Caucasian salamander *Mertensiella caucasica* (Waga, 1876) *sensu lato*. Some of these organisms are limited to only a small part of the Caucasus, while others have much wider ranges (Myers *et al.* 2000; Tarkhnishvili 2014). Georgia covers less than 20% of the Caucasus, but it lies in the central parts of the ecoregion, encompassing all the landscapes stretching from the peat bogs in the west through the semi-deserts to the east and high mountains to the north. The only native crayfish species in Georgia is *Astacus colchicus* Kessler, 1876. It has not often been mentioned in the literature, and the species description was based on specimens gathered in the upper tributaries of the Rioni River (Kessler 1876). Much later, Bott (1950), Albrecht (1982), and Starobogatov (1995) compared species specific characteristics with other *Astacus* species/subspecies, presenting descriptive figures or a simple dichotomous key. The distribution of this species is known to be restricted to the upper Rioni basin in western Georgia (Kessler 1876; Albrecht 1982). Although Holthuis (1961) also reported *A. colchicus* from northern Turkey (in a creek close to Ünye town), these crayfish were determined to be narrow-clawed crayfish *Pontastacus leptodactylus* (Eschscholtz, 1823) later on (Machino & Holdich 2006). Another east Ponto-Caspian crayfish thick-clawed crayfish *Pontastacus pachypus* Rathke, 1837, is also known from southern slopes of the eastern Great Caucasus (Azerbaijan) and was suggested to occur in Georgia though it has never been recorded there (Derzhavin 1951) or having disappeared as in most of area of its occurrence in Ukraine (Policar *et al.* 2018).

The systematics of European freshwater crayfish underwent significant improvement due to advanced molecular tools, with most of the studies dedicated to *Austropotamobius* species (Zaccara *et al.* 2004; Trontelj *et al.* 2005; Klobučar *et al.* 2013; Jelić *et al.* 2016; Pârvulescu 2019; Pârvulescu *et al.* 2019) and some to the noble crayfish *Astacus astacus* (Linnaeus, 1758) (Bláha *et al.* 2016; Laggis *et al.* 2017; Schrimpf *et al.* 2017). On the other hand, Eastern European species are still somewhat inaccessible and suffer from a lack of data; their taxonomy

relying mostly on morphological data and historical records and their systematics still not fully resolved (however see, Maguire *et al.* 2014; Akhan *et al.* 2014; Bláha *et al.* 2017). Recently, an updated classification of the freshwater crayfish was published (Crandall & De Grave 2017), keeping the rich taxonomic nomenclature of European crayfish as suggested by Karaman (1962), Albrecht (1982), or Starobogatov (1995) with respect to species and subspecies within the genera *Astacus* (3 species and 2 subspecies) and *Pontastacus* (9 species and 1 subspecies), respectively. This recent study should minimize the differences in nomenclature used by some authors (Kouba *et al.* 2014; Maguire *et al.* 2014; Šmietana *et al.* 2006) and uncertainty as to which taxon name should be correctly used. On the other hand, some of these species and subspecies are defined solely based on morphological traits and/or zoogeography, but not tested with modern molecular or morphometric tools. It would help to exclude that these differences in morphology are not resulting only from high intraspecific variability and phenotypic plasticity of the species. One of such cases is that of *A. colchicus*. Although the main morphological differences from *A. astacus* were already mentioned (Karaman 1962; Albrecht 1982; Starobogatov 1995), those differences could be a consequence of morphological plasticity and not really species specific. Recently, a new species *Austropotamobius bihariensis* Pârvulescu, 2019 has been described from the Apuseni Mountains in Romania (Pârvulescu 2019). Morphological differences from the closest relatives were in the shape of the rostrum or antennal scale, that is, differences which could be easily overlooked and originally considered within the phenotypical plasticity of the *Austropotamobius torrentium* (Schrank, 1803). However, the author of the species description found high genetic divergences from other *A. torrentium* populations and then applied detailed morphometry to find significant differences between the new species and its closest relatives.

No molecular genetic methods have so far been applied to *A. colchicus*, and no relevant data exist about its genetic diversity, phylogenetic position, and morphometry. Therefore, we present here the molecular and morphological analysis of the *A. colchicus* sampled in Georgia with 2 main aims: (i) to provide morphological and genetic data for this species, and (ii) to describe its phylogenetic position and reveal whether it represents a separate lineage to *A. astacus* or is clustered within *A. astacus* species and thus any morphological differences should be accounted as high intraspecific variability only.

MATERIALS AND METHODS

Specimen collection

In total, 106 crayfish individuals from 10 sampling sites (Table 1; Fig. S1, Supporting Information) in Georgia were collected by hand or trapping during 2016. Individuals of *A. colchicus* ($n = 51$) were identified in only 6 of them (Tables S1 and S2, Supporting Information), *P. leptodactylus* occurred in the rest of sampling sites as well as was found in sympatry with *A. colchicus* at Sepa river. Each of the 6 sampling sites were located in the catchments of Churia (Papantskuri Lake), Khobi, Rioni (Lashe river), Sepa, Choloki, and Kintrishi rivers. One pereopod from each animal was dissected and individually preserved in pure 96% ethanol until DNA extraction. Most individuals were released back at the locality and allowed to regenerate.

Morphometric analysis

Morphological analysis of a total number of 51 individuals of *A. colchicus* was carried out with a total number of 21 morphological characteristics recorded for each crayfish, following Sint *et al.* (2005). Particular characteristics were measured with an electronic caliper to the nearest 0.1 mm. Any injured, damaged and regenerated claws were not used for measurements. All measurements were inverted for a 17 indices: CPL/CLL—length of the claw palm to the claw length; CLW/CLL—claw width to the claw length; HEL/TL—head length to the total length; CEW/TL—width of the carapace at the hind edges to the total length; CPW/TL—carapace width to the total length; ABW/TL—abdomen width to the total length; ABH/TL—abdomen height to the total length; TEW/TL—telson width to the to the total length; ROL/TL—rostrum length to the total length; CLH/CLW—claw height to the claw width; CFL/CPL—length of the claw finger to the length of the claw palm; TEL/TEW—telson length to the telson width; ROL/ROW—rostrum length to the rostrum width; ABL/TL—abdomen length to the total length; CPX/TL—carapace length (rostrum length, head length, areolar length are included) to the total length; CPX/CPW—carapace length (rostrum length, head length, areolar length are included) to the carapace width; HEL/HEW—head length to the head width. Further, individuals of *A. astacus* ($n = 100$), originating in Podolský brook, Vápenný Podol village, Czech Republic, were also measured and analyzed to contrast morphological differences between species. Multivariate redundancy analysis (RDA) was performed to describe differences between *A. colchicus* and *A. astacus* using the software Canoco

version 5.0 (ter Braak & Šmilauer 2012). Monte-Carlo permutation test (4999 permutations, blocks defined by covariates) was applied for testing significance of the RDA model, that is, differences in morphometrics between both species, with sex as a covariate. Analysis of covariance (ANCOVA), run in Statistica 12 (StatSoft Inc.), was used to compare differences of individual morphometric indices between 2 species with sex as a covariate as well. Since some of the data did not have normal distribution (tested by Shapiro–Wilks test), Box-Cox transformation was applied. Supplementary pictures of body habitus, abdominal pleura, and carapace were done using a male individual from Khobi river and a female from Lashe river.

Morphological characteristics described by Füreder and Machino (2002) and keys to palaeartic fauna (Rogers & Thorp 2019) were used to genera determination, while study of Albrecht (1982) and Starobogatov (1995) to check specific differences of *A. astacus*.

Molecular data collection

Genomic DNA extraction and PCR amplification was done according to Bláha *et al.* (2016) using 36 *A. colchicus* individuals. Three mitochondrial genes, cytochrome c oxidase I (COI), 16S and 12S rRNA, and nuclear histone H3 (H3) were applied (details in Table 2). Product purification and sequencing was performed by Macrogen Inc., Korea.

Phylogenetic analysis

All newly obtained sequences were deposited in GenBank under accession numbers listed in Table 1. Sequences were aligned with MAFFT version 7 (Katoh *et al.* 2002) implemented in GENEIOUS version 8.0.5 (www.geneious.com; Kearse *et al.* 2012); COI and H3 alignments were translated into amino acids to check for indels and stop codons. Analysis of synonymous and non-synonymous substitutions were done in DnaSP version 5.10.01 (Librado & Rozas 2009) to omit usage of pseudogenes. Pairwise model-corrected genetic distances were calculated for each gene in PAUP* version 4.02b (Swofford 2001), for which we report the mean genetic distance in order to compare the relative amounts of divergence of each gene and among species. In addition to the *A. colchicus* samples from Georgia, available sequences of 5 Astacidae species [*A. astacus*, *P. leptodactylus*, *P. pachypus*, *Austropotamobius pallipes* (Lereboullet, 1858), and *A. torrentium*] as an ingroup, and *Pacifastacus leniusculus* (Dana, 1852) as an outgroup

Table 1 Information on sampling locality, type of habitat, geographic coordinates, number of analyzed individuals (*N*), and GenBank accession number of particular haplotypes (H) together with GenBank accession number of other species used in this study

Locality	Habitat type	Geographic coordinates	COI		16S		12S		H3					
			<i>N</i>	H	GenBank Acc. number	<i>N</i>	H	GenBank Acc. number	<i>N</i>	H	GenBank Acc. number			
<i>Astacus colchicus</i>														
Lashe river, Tkemlovani	Small, slow flowing mountain river	42°11'12.43"N 43°18'28.65"E	11	3	MT483861, MT483865	7	4	MN809183- 186	11	2	MN809190, MN809194	6	2	MT237558, MT237559
Papantskuri Lake, Sakirio	Lake	42°22'26.1"N 1°49'50.0"E	9	2	MT483860, MT483862	9	2	MN809187, MN809189	8	1	MN809191	5	2	MT237559
Khobi river, Khobi	Big river	42°19'46.4"N 41°54'10.4"E	1	1	MT483864	1	1	MN809188	1	1	MN809198	1	1	MT237560
Tributary of Choloki river, Kakuti	Small brook	41°51'53.3"N 41°56'34.9"E	6	1	MT483863	6	1	MN809187	6	2	MN809192, MN809196	4	2	MT237559, MT237561
Kintrishi river, near Nakaidzeebi	Medium size mountain river	41°48'14.4"N 41°48'12.2"E	1	1	MT483860	1	1	MN809187	1	1	MN809197	1	1	MT237559
Sepa river, near Ekaldidi	Medium size lowland river	41°59'06.4"N 41°49'03.0"E	9	1	MT483860	3	1	MN809187	6	2	MN809193, MN809195	4	3	MT237558- 560
<i>Astacus astacus</i>														
<i>Pontastacus leptodactylus</i>														
<i>Pontastacus pachypus</i>														
<i>Austropotamobius torrentium</i>														
<i>Austropotamobius pallipes</i>														
<i>Pacifastacus leniusculus</i>														

Table 2 Primer sequence used for amplification with annealing temperatures

Primer	Sequence (5' – 3')	Annealing temperature (°C)	Source
LCO 1490	GGTCAACAAATCATAAAGATATTGG	50	Folmer <i>et al.</i> (1994)
COI 703r	CCRCCMGCAGGRTCAAAGAA		This study
16S ar	CGCCTGTTTAACAAAAACA	55	Simon <i>et al.</i> (1994)
16S br	CCGGTCTGAACTCAGATCACGT		
16S brAst	CCGGTRTGA ACTCAGATCACGT		This study
12S F5357	ATYTTGTGCCAGCAGTCGCG	61	This study
12S R5937	CTTAAATGAAAGCGACGGGC		
H3 AF	ATGGCTCGTACCAAGCAGACVGC	50	Colgan <i>et al.</i> (1998)
H3 AR	ATATCCTTRGGCATRATRGTGAC		

corresponding to the COI, 16S and 12S rRNA mitochondrial genes, and nuclear H3 genes were downloaded from NCBI's GenBank (Table 1). Therefore, phylogenetic relationships were reconstructed using the concatenated dataset from 7 crayfish species. The final length of particular sequences used for alignment was 648 bp for COI, 489 bp for 16S, 471 bp for 12S, and 327 bp for H3 or 1935 bp in concatenated alignment. jModel Test 2.1.7 (Darriba *et al.* 2012) was used to find the optimal model of substitution for a particular gene based on Bayesian information criterion. The optimal models found for COI, 16S, 12S, and H3 alignment were HKY + G, TPM1uf + I, HKY + G, and K80, respectively. A maximum likelihood (ML) tree was constructed in RAxML version 7.2.871 implemented in GENEIOUS, with each partition having its own GTRGAMMA model, and nodal support of the tree was tested via 2000 bootstrap replicates. Bayesian analyses were conducted in MrBayes 3.2.4. (Ronquist *et al.* 2012) applying the specific nucleotide substitution model for a particular gene sequence set. The generated log files were analyzed with TRACER (Rambaut *et al.* 2013) to confirm that effective sample size values were >200 for all parameters, and that stationarity between particular runs was ensured after the burn-in period.

RESULTS

Morphology

All 51 analyzed individuals of *A. colchicus* demonstrated following characteristics clearly indicating affiliation with genus *Astacus*: male pleopod II without ventral process (talon) and abdominal somites II and III with pleura rounded or angular, lacking spines. Individuals of *A. colchicus* demonstrated also more rounded abdominal somites in comparison to *A. astacus*, which have

abdominal somites wedge-shaped (Fig. S2, Supporting Information). *Astacus colchicus* had well-developed posterior postorbital ridges, approximately 2 times longer than anterior ones and posteriorly curved inward (Fig. S3, Supporting Information). Details about morphometry and sex of individuals are shown in the Tables S1 and S2, Supporting Information. Habitus of the crayfish can be seen in Fig. S4, Supporting Information.

RDA analysis (Fig. 1) explained 28.43% of variation. Differences found between 2 analyzed species were highly significant ($P = 0.001$, F -like statistic value = 53.0). Comparison of individual indices revealed almost all indices differed significantly except ABW/TL, TEW/TL, CLH/CLW, TEL/TEW, and ABL/TL (Table S3, Supporting Information).

Sequence data and phylogenetic analysis

From 36 analyzed specimens of *A. colchicus*, 36 sequences were recovered for COI, 27 sequences for 16S, 33 sequences for 12S, and 21 sequences for H3 (Table 3). The combined mitochondrial and nuclear dataset consisted of 16 haplotypes of the ingroup (11 of *A. colchicus*, 1 of *A. astacus*, *P. leptodactylus*, *P. pachypus*, *A. torrentium*, and *A. pallipes*, respectively), and 1 haplotype (*P. leniusculus*) of the outgroup.

The mean model-corrected sequence distances among *A. colchicus* and *A. astacus* were 10.9% for COI, 9.6% for 12S, 6.5% for 16S, and 1.1% for H3, while mostly similar distances were recorded for *P. leptodactylus* or *P. pachypus* (Tables S4 and S5, Supporting Information).

All the combined mtDNA and nDNA phylogenetic analyses recovered sequences of *A. colchicus* comprising a monophyletic clade with high statistical support. The other 6 monophyletic clades represented rest of species

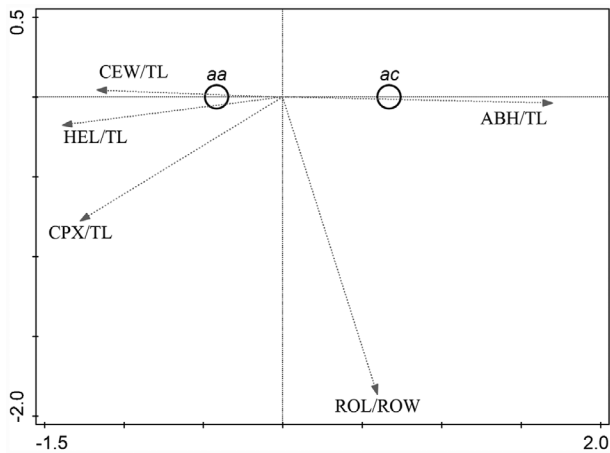


Figure 1 Morphometric characteristics-species biplot of RDA on standardized functional trait data. This diagram summarizes the variation in morphometric composition explained by species, after accounting for the effects of covariates (sex). The first 5 morphometric indices with highest fit are shown by given arrows and labeled by particular abbreviations (ABH/TL abdomen height to body total length, HEL/TL head length to body total length, CPX/TL carapace length to body total length, CEW/TL carapace width to body total length, and ROL/ROW rostrum length to rostrum width). The centroids of species are indicated by black empty circle (aa *Astacus astacus*, ac *A. colchicus*). The distance between the species centroids approximates the average dissimilarity of morphometric composition between these two species being compared as measured by their Euclidean distance

used in the analysis also with high statistical support in Bayesian analyses (Fig. 2). *Astacus colchicus* was shown to be a sister clade to *P. leptodactylus* and *P. pachypus*.

DISCUSSION

Our molecular and morphometric analysis revealed and indicated a unique phylogenetic and morphometric pattern of *A. colchicus* populations from Georgia, and corroborated correct taxonomic assignment to genus

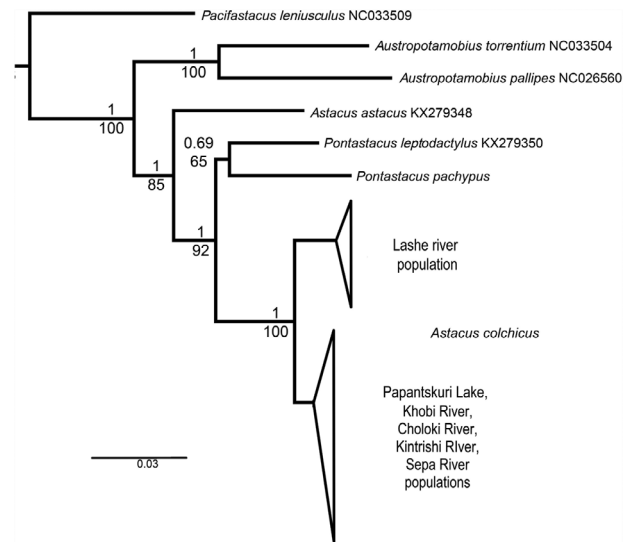


Figure 2 Bayesian tree reconstruction based on concatenated data set. Bayesian inference and Maximum likelihood bootstrap are displayed above and under each node, respectively

Astacus and species status as well. Despite a high morphological similarity with *A. astacus*, morphometric analysis revealed several characteristics useful for the differentiation from this species, namely ABH/TL, HEL/TL CPX/TL, CEW/TL, and ROL/ROW showing the most obvious differences (Fig. 1; Tables S1 and S2, Supporting Information). The different ratio of head length comparing to carapace or total length could be easily recognized from the drawings of Albrecht (1982) or pictures of these two crayfish species presented in this study (Figs S2–S4, Supporting Information). Besides characteristics dependent on measuring of individuals and calculating the particular indices, for most field researchers, there are also several distinguishing morphological characteristics without need of measuring or keeping the animals for necessary time. Especially, shape and length of posterior pair of postorbital ridges and shape of abdominal somites are well distinctive (Albrecht 1982; Starobogatov 1995).

Table 3 Nucleotide polymorphism of *Astacus colchicus* sequences based on mitochondrial (COI, 16S, 12S) and nuclear (H3) data

Gene	Length (bp)	VS	PI	N	H	Hd (SD)	π (SD)
COI	648	24	22	36	8	0.862 (0.022)	0.015 (0.002)
16S	484	7	5	27	7	0.598 (0.108)	0.004 (0.001)
12S	465	24	21	33	9	0.856 (0.030)	0.018 (0.001)
H3	327	2	2	21	4	0.628 (0.092)	0.002 (0.001)

VS, number of variable sites; PI, number of parsimony informative sites; N, number of sequences used; H, number of haplotypes determined; Hd, haplotype diversity; π , nucleotide diversity.

Although sympatry of these 2 species is improbable regarding mainly to endemism of *A. colchicus*, described morphological characteristics could be useful especially when analyzing old museum samples. The characteristics used to distinguish genera *Astacus* and *Pontastacus*, the shape of abdominal somites being with acute spines at their ventral ends and presence of abdominal process in second male pleopod in *Pontastacus*, are simply summarized in Rogers and Thorp (2019) or Füreder and Machino (2002). Furthermore, comparing to *P. leptodactylus*, *A. colchicus* has immovable finger of chela with incision in median part of inner margin, with *P. leptodactylus* having no such an incision. Sometimes, the incision of *Astacus* could be only weakly expressed; then there are clearly visible tubercles at the end of immovable finger. All these characteristics seem to be solid enough across a wide area of occurrence of both genera/species.

The results of molecular study clearly indicate deep molecular divergence with relatively high molecular distance for particular genes (Tables S4 and S5, Supporting Information). Although the high morphological similarity with *A. astacus* has led some scientists to assign the populations from Georgia as its subspecies (Albrecht 1982; Bott 1950), the others correctly appraise all indicia to assign it to valid species status (Karaman 1962; Starobogatov 1995; Crandall & De Grave 2017). At the same time, it automatically brings up a question about the phylogenetic position and species status of *A. balcanicus balcanicus* Karaman, 1929 populations, also presented by the authors of the recent updated classification of freshwater crayfishes as a valid species (Crandall & De Grave 2017). This species has a similar status, being morphologically very similar to *A. astacus* and by occurrence restricted to the area of the Vardar river system (Greece, Macedonia) and Ohrid Lake (Macedonia) (Albrecht 1982, 1983). Recently, Laggis *et al.* (2017) have analyzed *A. astacus* populations from Greece (thought to be *A. balcanicus*) at the southernmost area of the species distribution and identified 2 new phylogroups different from other known European ones. However, molecular distances recorded were much lower (up to 4.1% for COI and 1.9% for 16S) compared to those recorded between *A. colchicus* and *A. astacus* in our recent study, so not suggesting species status (Laggis *et al.* 2017). The *A. balcanicus* issue could hopefully be resolved by sampling and genetic analysis of *Astacus* species from the type locality (Ohrid Lake). This lake harbors many endemic organisms; however, the only crayfish species is referred to as *A. astacus* and its population density is quite low (Albrecht & Wilke 2008). Nevertheless, the past history of this area in Europe is very rich in geolog-

ical processes (the Alpine–Carpathian–Dinaric orogeny) affecting the establishment of many aquatic species including crayfish (Copilaş-Ciocianu & Petrusek 2015; Mráz & Ronikier 2016; Pârvulescu 2019; Pârvulescu *et al.* 2019). Moreover, the Balkan region is considered one of the major glacial refugia for many species during the Pleistocene climatic oscillations (Hewitt 2004), its high genetic diversity of species later spreading to the rest of the unglaciated areas. A further revision of particular species/subspecies within *Astacus* is still needed to clarify the taxonomy of this dominant European crayfish taxon.

The high molecular divergence between *A. colchicus* and *A. astacus* is most likely caused by past paleogeographical events in the Ponto-Caspian region and thus a relatively long-time separation. Regarding the fauna of the Caucasus and their evolutionary relationships to other European relatives, there is a certain pattern driven by climatic and landscape changes shaping the establishment of a new species (Tarkhnishvili 2014). The earliest range fragmentation between the Caucasus, Western Europe, and Mediterranean area was linked to the early and middle Miocene (22–13 Mya) (Popov *et al.* 2004). This event caused a split between the *Mertensiela caucasica* and its closest relatives *Chioglossa lusitanica* Bocage, 1864 (Veith *et al.* 1998; Weisrock *et al.* 2001) as well as a split between *P. caucasicus* and its closest western European relative, *Pelodytes punctatus* (Daudin, 1802) (Garcia-Paris *et al.* 2003; Veith *et al.* 2006). Further Miocene–Pliocene range fragmentation linked to the Messinian salinity crisis (ca. 6 Mya) resulted in a global decline of humidity, environment instability and further landscape fragmentation, and finally, middle and late Pliocene range fragmentation after the Messinian salinity crisis and before the first glacial waves (Tarkhnishvili 2014). It caused a later separation between Caucasian populations of *Lisotriton vulgaris lantzi* (Krasavtzev, 1940) and its European populations (Babik *et al.* 2005) as well as between *Rana macronemis* Boulenger, 1885, and its closest western European relatives (*Rana* group, Veith *et al.* 2003). All of these events caused a decline in temperature followed by declines of evaporation and precipitation, resulting in landscape fragmentation (Zachos *et al.* 2001; Tarkhnishvili 2014), which might also have substantial effects for the origin of *A. colchicus*. Consequently, repeated isolation/connection between ancient Balkan and Anatolian (Pontides) lands throughout the Miocene could be thought as a basis for split between ancestors of *A. astacus* and eastern Ponto-Caspian crayfish clade, while a subsequent orogeny and range fragmentation in Anatolian/Caucasian areas might cause a divergence between *A. colchicus* and other *Pontastacus* lineages.

Nevertheless, it is still not easy task to suggest the most likely scenario about the origin of *A. colchicus* as well as of other European crayfish species without understanding the context of European crayfish species evolutionary relationships. Moreover, depending on the methods applied, recent studies using significant part of the European crayfish species have resulted in quite a wide frame of their origin, encompassing the period from the Cretaceous (Porter *et al.* 2005; Toon *et al.* 2010; Bracken-Grissom *et al.* 2014) to the Miocene (Klobučar *et al.* 2013; Jelić *et al.* 2016). Pârvulescu *et al.* (2019) pointed out discrepancies between age estimates based on molecular clocks, using common standard arthropod substitution rates for mtDNA genes (Knowlton & Weigt 1998; Schubart *et al.* 1998), and those originating from applying fossil calibrations or paleogeographic events (Porter *et al.* 2005; Breinholt *et al.* 2009; Bracken-Grissom *et al.* 2014; Pârvulescu *et al.* 2019). According to recent studies (Parham *et al.* 2012; Warnock *et al.* 2015), usage of proper fossil calibration or paleogeographic events is the most suitable way to obtain the most realistic age estimates of particular nodes in a time tree. However regarding European crayfish history, only a few available fossil records exist (Garassino 1997; Taylor *et al.* 1999; Rode & Babcock 2003). Moreover, most of them have a too unclear taxonomic status to be obvious what current species are their descendants (Rode & Babcock 2003; Karasawa *et al.* 2013) or do not fit into current theories about the origin of European crayfish (Buscalioni & Poyato-Ariza 2016). Therefore, disentangling the origin and history of European or, in the more general context, of Northern Hemisphere crayfish is a challenging task requiring advanced analysis of most of the European crayfish species and careful choice of appropriate calibrations for age estimates.

CONCLUSION

This study provides a novel insight into European crayfish phylogeography including the Caucasian endemic crayfish *A. colchicus*. Both morphological and molecular analyses corroborated valid species status of the populations from Georgia and mentioned helpful characteristics used for species identification. Moreover, molecular part, comprising most of the European crayfish species in the genera *Astacus* and *Pontastacus*, has also resulted in different topology compared to previous studies using assemblages of fewer European species. The phylogenetic position of *A. colchicus* together with its zoogeography matches current ideas about the origin of European crayfish species. Future studies should aim at revision of *A. balcanicus* morphology and phylogenetic

position. Furthermore, specific information is still missing with respect to the status of rich taxa assigned to Eastern European crayfish *Pontastacus*, mostly characterized morphologically without molecular methods.

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SUPPLEMENTARY MATERIALS

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Map of Georgia showing sampling sites during the crayfish survey. Red full circle indicates population of *Astacus colchicus*, orange triangle - sympatric population of *A. colchicus* and *Pontastacus leptodactylus* while green square - population of *P. leptodactylus*.

Figure S2 Pleura of abdominal somites of *Astacus colchicus* male (A), female (B) and *A. astacus* male (C).

Figure S3 Carapace of *Astacus colchicus* (A) and *A. astacus* (B) male.

Figure S4 Habitus of *Astacus colchicus* male (A), female (B) and *A. astacus* male (C).

Table S1 Comparison of the morphometric indices of *Astacus astacus* and *A. colchicus* individuals with mean values and standard deviation (SD)

Table S2 Comparison of the morphometric indices of *Astacus astacus* and *A. colchicus* individuals with mean values and standard deviation (SD)

Table S3 ANCOVA results for comparison of particular morphometric indices between *A. colchicus* and *A. astacus*

Table S4 Mean model corrected sequence distances among species

Table S5 Mean model corrected sequence distances among species

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