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First record of the stone crayfish in the Romanian lowlands

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Abstract – The stone crayfish is a native European freshwater species with significant population declines caused by anthropogenic pressure onto its habitats, climate change and spreading of non-indigenous invasive crayfish and their pathogens (*e.g.*, *Aphanomyces astaci*). We present the first record of an *Austropotamobius torrentium* population in a small stream from the southern lowlands of Romania, in an area without limestone or karst, known as a typical stone crayfish habitat in this country. No additional populations were found in the neighbouring streams. The mtDNA analysis based on sequencing a short fragment of the cytochrome c oxidase subunit I (*COI*) gene revealed that specimens from this newly discovered population belongs to the most widespread phylogroup of *A. torrentium* (CSE) and share the same haplotype as the specimens found in Croatia. Unfortunately, this crayfish population has disappeared most likely because of the crayfish plague since pathogen *A. astaci* was isolated from moribund crayfish. Our study highlights the vulnerability of isolated relic populations in the face of outside stressors.

Keywords: *Austropotamobius torrentium* / *Aphanomyces astaci* / karst

Résumé – **Premier signalement de l'écrevisse des torrents dans la plaine roumaine.** L'écrevisse des torrents est une espèce d'eau douce européenne indigène dont les populations connaissent un déclin important en raison de la pression anthropique sur ses habitats, du changement climatique et de la propagation d'écrevisses invasives non indigènes et de leurs agents pathogènes (par exemple, *Aphanomyces astaci*). Nous présentons le premier enregistrement d'une population d'*Austropotamobius torrentium* dans un petit ruisseau des basses terres du sud de la Roumanie, dans une zone sans calcaire ni karst, connue comme un habitat typique de l'écrevisse des torrents dans ce pays. Aucune autre population n'a été trouvée dans les ruisseaux voisins. L'analyse de l'ADNmt basée sur le séquençage d'un court fragment du gène de la sous-unité I de la cytochrome c oxydase (COI) a révélé que les spécimens de cette population nouvellement découverte appartiennent au phylogroupe le plus répandu d'*A. torrentium* (CSE) et partagent le même haplotype que les spécimens trouvés en Croatie. Malheureusement, cette population d'écrevisses a disparu très probablement à cause de la peste des écrevisses depuis que l'agent pathogène *A. astaci* a été isolé des écrevisses moribondes. Notre étude met en évidence la vulnérabilité des populations relictuelles isolées face aux facteurs de stress extérieurs.

Mots-clés : *Austropotamobius torrentium* / *Aphanomyces astaci* / karst

Freshwater crayfish are an old group of invertebrates, with different adaptive potentials (Reynolds *et al.*, 2013). They generally inhabit fresh waters with heterogeneous substrate that provides shelters (Füreder *et al.*, 2006), and are mostly negatively influenced by organic and inorganic pollutants (Reynolds *et al.*, 2013). Climate change, deforestation, habitat deterioration and introduction of the alien invasive crayfish species to their environment negatively affect their chances of survival (Holdich *et al.*, 2009).

The stone crayfish (*Austropotamobius torrentium*) is one of the most threatened crayfish species in Europe (Streissl and Hodl, 2002), mostly due to human impact onto their natural habitats (Maguire *et al.*, 2018). This species is a complex of genetically divergent phylogenetic groups (Klobučar *et al.*, 2013; Pârvulescu *et al.*, 2019; Lovrenčić *et al.*, 2020), with typically high environmental requirements. It is most commonly present in cold (Souty-Grosset *et al.*, 2006), well-oxygenated waters (Pârvulescu *et al.*, 2011), with moderate current velocities (Pockl and Streissl, 2005). It mostly prefers habitats with hard substrate (Bohl, 1988) and a

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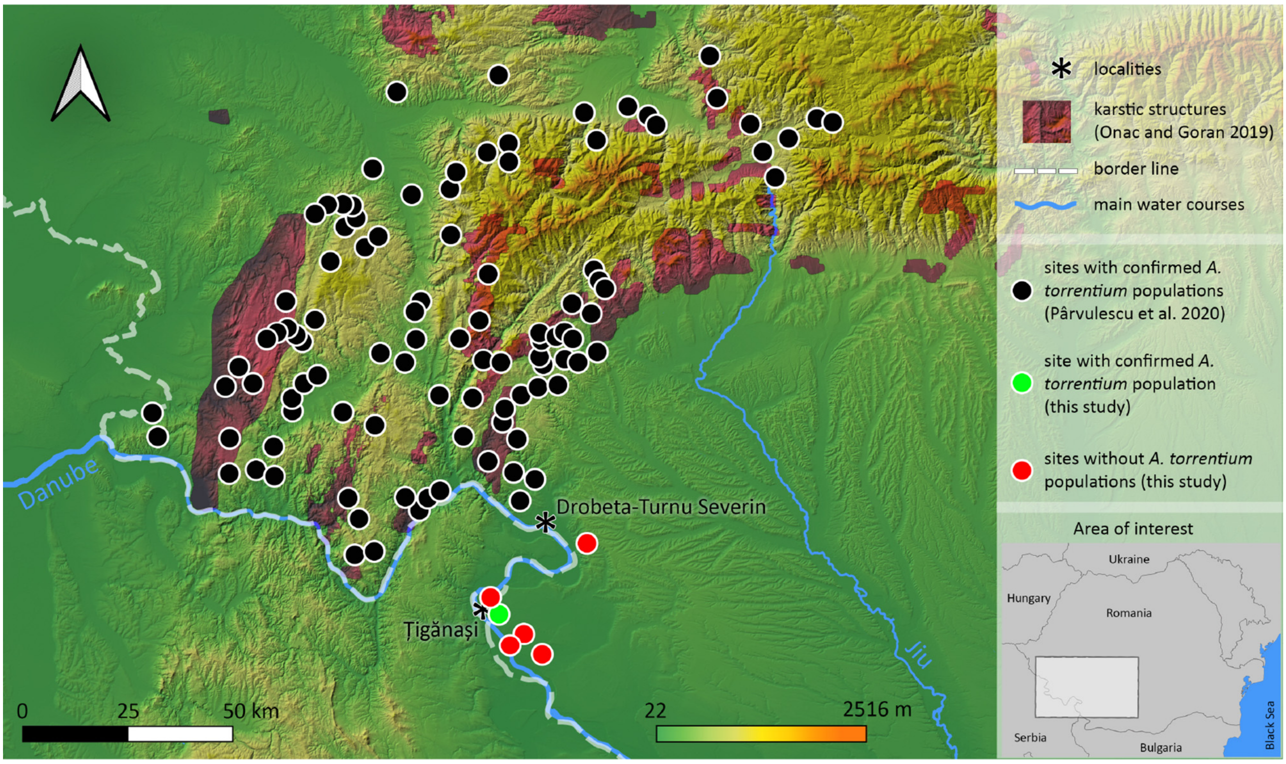

Fig. 1. The map was made using QGIS 3.16.1-Hannover version and GIMP 2.10.22 version. It was designed by the superimposition of the *A. torrentium* distribution map from Pârvulescu *et al.* (2020) and the karstic structures map from Onac and Goran (2019).

high number of suitable stone shelters (Vlach *et al.*, 2009; Pârvulescu *et al.*, 2016). Due to their environmental requirements and negative anthropogenic pressure onto their habitats, this species has a fragmented dispersal across its distribution range (Kouba *et al.*, 2014). According to recent studies (Klobučar *et al.*, 2013; Pârvulescu *et al.*, 2019, Lovrenčić *et al.*, 2020), *A. torrentium* most likely began its spread from the Dinarids (in early Miocene) towards eastern and southern Europe *via* pre-glacial and post-glacial dispersal routes, later linked to the Danube River and its tributaries that enabled its dispersal into central Europe (Klobučar *et al.*, 2013), reaching its northernmost limits of distribution in the Rhine and Elbe River Basins (Petrusek *et al.*, 2017).

Previous molecular studies established that the population of the stone crayfish in the south-western part of the Carpathians in Romania belongs to the central-south European (CSE) phylogroup *sensu* Klobučar *et al.* (2013). Stone crayfish migrated to that region *via* the Danube River system (Pârvulescu *et al.*, 2019; Lovrenčić *et al.*, 2020), and survived Pleistocene glaciations due to suitable refuge habitats (Pârvulescu *et al.*, 2013). Currently, these populations' distribution is limited to mountainous and sub-mountainous karstic regions (Pârvulescu *et al.*, 2013, 2020). Presently, their survival is confined by the presence of co-occurring species: *Astacus astacus* and recently introduced *Faxonius limosus* (Pârvulescu *et al.*, 2009, 2011). The later species, is also a carrier and a spreading vector of the pathogen *Aphanomyces astaci* that is a causative agent of crayfish plague (Kozubíková *et al.*, 2011), disease that is most frequently lethal for native crayfish species and subsequently poses one of the most serious threats to their survival (Jussila *et al.*, 2017). *Faxonius limosus* carrying the pathogen has colonised a long stretch of the Danube River in Romania (Pârvulescu *et al.*, 2012), but, to our knowledge, there is no published evidence about its contact with the populations of *A. torrentium* in the small tributaries of the Danube, nor is there any mention of the *A. astaci* infection within the stone crayfish populations from the aforementioned streams.

In this study, we present the first report of an *A. torrentium* population in the lowlands of Romania (an altitude of 40–50 m/asl). This population was discovered in 2018 in a small stream, in the Blahniţa Plain, near Ţigănaşi Village, approximately 50 km away from the mountains and the karstic habitats (Fig. 1). This stream is a small tributary of the Danube River and is connected to a series of irrigation channels. It flows into the Danube River 2.2 km downstream from Ţigănaşi Village (Fig. 2). The last 500 m of the stream (section WSWC in Fig. 2), close to its mouth, possess conditions similar to typical stone crayfish habitat, with coarse sand and gravel, small to medium sized stones as a substrate and many larger stones, randomly spread throughout the stream. Upstream part of this stream is different (section WSHC in Fig. 2); a smoother substrate of sand with silt and steeper banks with tree roots rising from the water. This, approximately 4.5 km stretch of the stream (sections: WSWC, WSHC, Fig. 2), is surrounded by an alder grove. The crayfish searches were made only in the last two downstream sections (WSWC, WSHC) because section BSWC, situated upstream from WSWC and WSHC, was

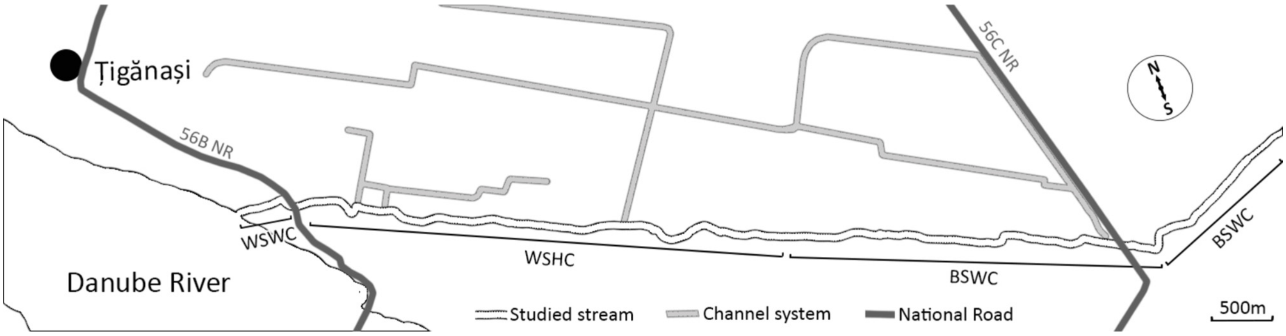

Fig. 2. The map of the studied stream containing the most important roads that pass over it and the manmade artificial channels it connects with; WSWC – wooded section without crayfish; WSHC – wooded section harbouring crayfish; BSWC – barren section without crayfish.

lacking tree coverage and was vastly affected by agriculture. Crayfish were caught only in the WSHC section of the stream with no recorded findings in the WSWC section, even though the conditions in WSWC section seem to be more suitable for this species. Five, apparently healthy looking, individuals and nine in poor health condition (limp or dead), were caught. The crayfish were captured by hand and were 7–10 cm long (total length, from the tip of the rostrum until the end of telson).

Samples (the distal segment of pereiopod five) for mtDNA *COI* analysis were taken from the healthy crayfish. Since crayfish in the poor health condition showed gross signs of crayfish plague (numerous melanisations on the carapace and pereiopods), we also sampled them/removed them from the stream for later laboratory analyses. Both sample types were preserved in the 95% alcohol until laboratory processing.

In order to establish crayfish phylogenetic position samples were processed and analysed according to procedure described in Lovrenčić *et al.* (2020). Obtained *COI* sequences were collapsed into already described haplotype AT108 that belongs to CSE phylogroup *sensu* Klobučar *et al.* (2013) (Appendix 1).

In order to test sampled tissues for *A. astaci* we followed procedure of Pavić *et al.* (2020). Analysis showed that sampled stone crayfish were infected by *A. astaci* and their poor health status and observed mass mortality was a consequence of crayfish plague outbreak. The pathogen load was level A2 what disabled genotyping (Vrålstad *et al.*, 2009).

The search for this *A. torrentium* population was repeated twice during October (mating time/period of crayfish high activity) 2020, but not a single crayfish was captured. Furthermore, 4 similar looking streams in the proximity were also thoroughly searched for the presence of the crayfish (Fig. 1), with the same outcome. Even though, three out of the four additional streams run directly into the Danube River (where *F. limosus* was observed - personal observations), there were no records of this invasive species within any of them. This absence of crayfish in all of these four additional streams is most probably linked to the fine grain substrate, the high velocity shallow water throughout most of the watercourses and the lack of suitable shelters or banks suitable for burrowing. Also, crayfish absence could have been a consequence of anthropogenic pressure onto the habitat since these streams are surrounded by settlements and agriculture land.

Guarding in mind previously adduced, a question that arises is: How did the population of *A. torrentium* get to the stream near Ţigănaşi? Their presence in Romania is related to their past migrations in the Miocene-Pleistocene period, when they also colonised the southern part of the Carpathians through the Danube River and its tributaries (Lovrenčić *et al.*, 2020; Pârvulescu *et al.*, 2020). Thus, the most probable explanation for their discovery away from the expected areas could be the close relation of this species to the Danube River system. Stone crayfish populations might have been widespread in the streams of this area, but only the Ţigănaşi population survived.

This claim is also supported by the molecular phylogenetic analysis (Appendix 1). This population is part of the CSE clade *sensu* Klobučar *et al.* (2013), together with all the stone crayfish populations within the southern Carpathians, in Romania (Pârvulescu *et al.*, 2020), and are also closely related to some populations in Croatia (Lovrenčić *et al.*, 2020). The Danube River changed its course throughout the Pleistocene period, starting from its most north-eastern location in the Günz ice age and reaching its most south-western position (the current location) after the last glaciation (Würm) (Răducă *et al.*, 2019). The Danube might have played an essential dualistic role in the spread of the crayfish, firstly, by acting as a refuge during the glaciations and secondly, by being a strong barrier in the migration path of the animals coming from the Carpathians, in the north. Thus, the *A. torrentium* population near Ţigănaşi might have been a relic of the past populations that found refuge in the lower and probably warmer area, of southern Romania. Similar patterns of settling into lower elevations with a milder climate, during the ice ages as a survival mechanism/strategy, were also recorded for numerous plant and animal taxa. For example, trees from genus *Fagus* can be found here, at lower altitudes than expected (Pascovschi, 1967). Also, species classified as mountainous, like some terrestrial isopods, *Hyloniscus transsilvanicus* and *Ligidium germanicum* (Ferenţi and Covaciu-Marcov, 2014) and *Salamandra salamandra* (Covaciu-Marcov *et al.*, 2017), were also found in the Blahniţa Plain region. Another important finding that supports our hypothesis is the presence of *Mesoniscus graniger* (Ferenţi and Covaciu-Marcov, 2018) in the Blahniţa Plain. This isopod species was thought to be related only to the caves in the mountainous karstic region, until it was discovered in the lowlands. The presence of *Darevskia praticola* in the open areas near streams of this region (Covaciu-Marcov *et al.*, 2009), is also very important, proving that these areas used to be covered with forests, that are known as factors that enable stable conditions in freshwater habitats (Beschta, 1991).

And yet we cannot rule out possibility that the Ţigănaşi population was of anthropogenic origin, but we could not find any written evidence in the literature that would support this hypothesis.

The presence of *A. astaci* in the studied stream was established, but pathogen level was too low and we could not determine its genotype. We can only presume that it belongs to Or genotype (haplogroup E) that is connected to the spiny-cheek crayfish (Kozubíková *et al.*, 2011) distributed in the vicinity (the Danube River). Infection could only have occurred recently, since *F. limosus* was seen in 2015 (Covaciu-Marcov, personal communication) at Portile de fier II, a village located on the bank of the Danube River, 13 km downstream from the studied stream. *Aphanomyces astaci* have reached Drobeta-Turnu Severin in 2014 (Pârvulescu *et al.*, 2015), and although there are no recently published updates on the plague spread/distribution, based on the distribution of the spiny-cheek crayfish, we can assume that the plague occurred at the same time as the invader near Ţigănaşi. The spread of the infection into the tributaries is not unexpected (Jussila *et al.*, 2015), taking into consideration that contact with any carrier of *A. astaci* zoospores (crayfish or fish) or fishing equipment could cause a crayfish plague outbreak (Schrimpf *et al.*, 2012; Maguire *et al.*, 2016).

A. torrentium individuals from the mouth of the stream, into the Danube River, might have come into contact with crayfish or other pathogen carriers and become infected first. Consequently, they also died first, explaining the lack of the crayfish in the WSWC section of the stream. The explanation for the lack of *F. limosus* in the stream may be linked to the size of the waterbody, as these crayfish were rarely seen entering and spreading upstream small tributaries (Petrusek *et al.*, 2006).

Our data indicated that *A. torrentium* probably had a historically wider distribution in Romania. Thus, we can infer that the populations here did not only survive in areas with limestone or karst during the Pleistocen glaciations (Pârvulescu *et al.*, 2013). It is possible that the studied population survived until 2018 only by chance, as they only occupied a segment of the stream (4.5 km long) that was not heavily affected by agricultural activity. But unfortunately, isolation, all the outside pressures and lastly, the crayfish plague have led to their demise.

Our report highlights the vulnerability of isolated relic populations in the face of outside stressors, both biotic (*A. astaci*) and abiotic (anthropogenic pressure onto the freshwater habitats) that frequently lead to their demise.

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Appendix 1

Phylogenetic tree of *A. torrentium* based on *COI* data obtained through Bayesian analysis performed in MrBayes 3.2.6 (Ronquist *et al.*, 2012). The optimal model of nucleotide evolution for *COI* data set was HKY + I + G, selected under the Bayesian information criterion (BIC) using the jModelTest 2.1.10. (Darriba *et al.*, 2012). The BA priors were set according to the suggested model; two separate runs with four Metropolis-coupled Monte Carlo Markov chains (MMCM) were performed for 10,000,000 generations, and trees were sampled every 1,000 generations. After checking congruence (ESS values > 200) with Tracer v1.7.1 (Rambaut *et al.*, 2018), the first 25% of sampled trees were eliminated as burn-in, and a tree was constructed, with nodal values representing the posterior probabilities. Numbers at the nodes indicate Bayesian posterior probabilities (values > 0.91 are indicated). New Romanian sequences belonging to haplotype AT108 are indicated in red colour. Phylogroups are represented by different colour: black – central and south-eastern Europe (CSE), blue – Gorski Kotar (GK), purple – Lika and Dalmatia (LD), orange – Žumberak, Plitvice and Bjelolasica (ŽPB), pink – southern Balkans (SB), green – Banovina (BAN), red – Zeleni Vir (ZV), gray – Apuseni Mountain (APU) and turquoise blue – Kordun (KOR).

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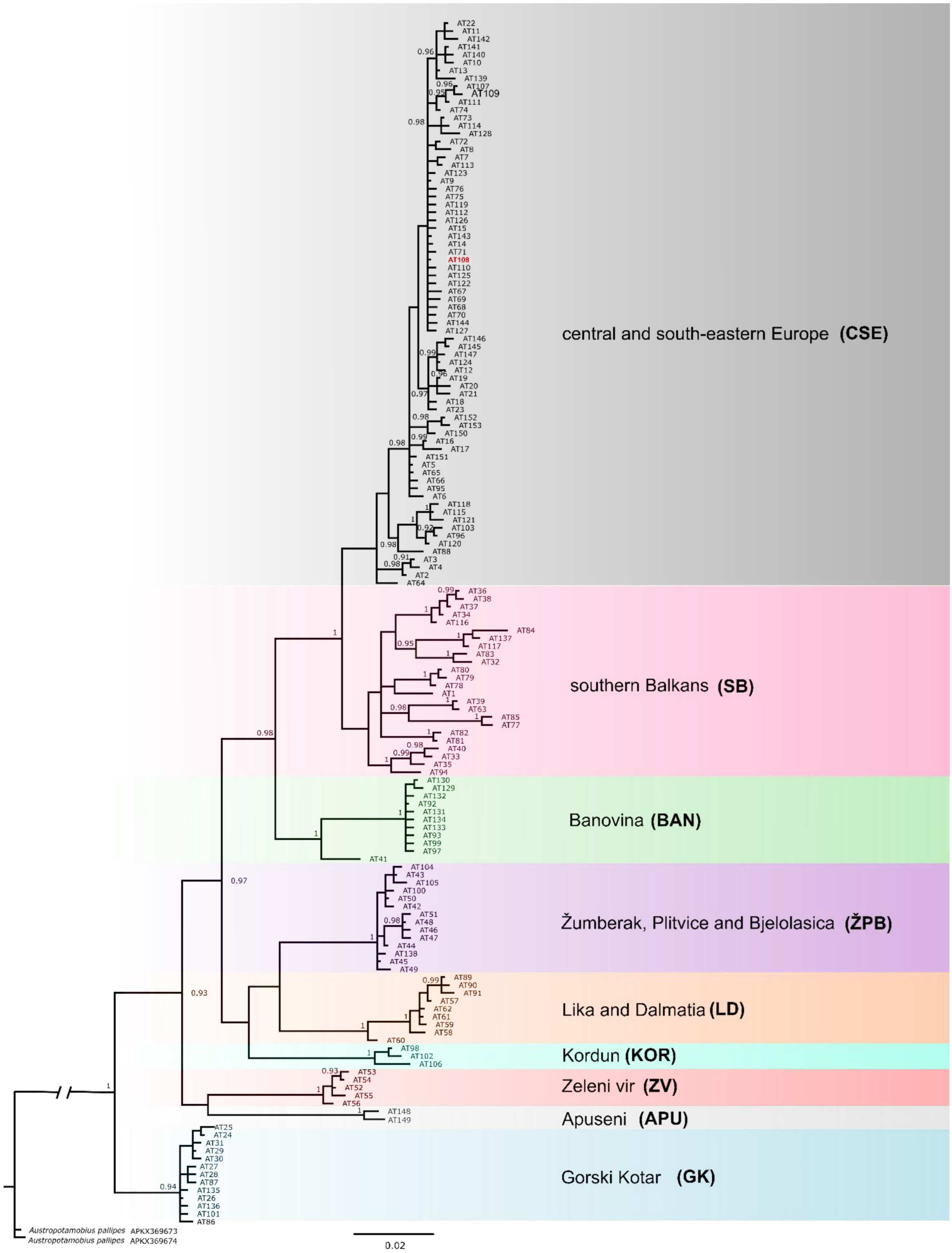
AT22
AT11
AT142
0.96
AT141
AT140
AT10
AT13
AT139
0.96
AT107
0.95
AT109
AT111
AT74
0.98
AT73
AT114
AT128
AT72
AT8
AT7
AT113
AT123
AT9
AT76
AT75
AT119
AT112
AT126
AT15
AT143
AT14
AT71
AT108
AT110
AT125
AT122
AT67
AT69
AT68
AT70
AT144
AT127
AT146
AT145
0.99
AT147
AT124
AT12
0.96
AT19
AT20
0.97
AT21
AT18
AT23
0.98
AT152
AT153
AT150
0.99
AT16
0.98
AT17
AT151
AT5
AT65
AT66
AT95
AT6
1
AT118
AT115
1
AT121
0.92
AT103
AT96
AT120
0.98
AT88
0.91
AT3
0.98
AT4
AT2
AT64
central and south-eastern Europe (CSE)
0.99
AT36
AT38
1
AT37
AT34
AT116
1
AT84
1
AT137
0.95
AT117
1
AT83
AT32
1
AT80
AT79
AT78
AT1
southern Balkans (SB)
1
AT39
0.98
AT63
1
AT85
AT77
1
AT82
AT81
0.98
AT40
0.99
AT33
1
AT35
AT94
0.98
AT130
AT129
AT132
AT92
1
AT131
AT134
AT133
AT93
AT99
AT97
1
AT41
Banovina (BAN)
AT104
AT43
AT105
AT100
AT50
0.97
AT42
AT51
AT48
0.98
AT46
AT47
1
AT44
AT138
AT45
AT49
Žumberak, Plitvice and Bjelolasica (ŽPB)
0.99
AT89
AT90
AT91
0.93
AT57
AT62
AT61
1
AT59
1
AT58
AT60
Lika and Dalmatia (LD)
1
AT98
AT102
AT106
Kordun (KOR)
0.93
AT53
AT54
1
AT52
AT55
AT56
1
Zeleni vir (ZV)
1
AT148
AT149
Apuseni (APU)
AT25
AT24
AT31
AT29
AT30
AT27
AT28
AT87
0.94
AT135
AT26
AT136
AT101
AT86
Gorski Kotar (GK)
Austropotamobius pallipes APKX369673
Austropotamobius pallipes APKX369674
0.02