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Haplotype variation in the *Physa acuta* group (Basommatophora): genetic diversity and distribution in Serbia

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Abstract

The genus *Physa* (= *Physella*) includes the most abundant and diverse freshwater gastropods native to North America. Due to their invasive nature, many species occur throughout the world. The most abundant species, *Physa acuta,* has been introduced to Europe, Africa, Asia and Australia by human commerce and migrating birds. This species is widely distributed throughout Serbia. The aim of this study was to explore the genetic diversity of *P. acuta* from Serbia, and to determine the evolutionary relationships among native Physidae populations from North America, Mexico and Cuba and populations from Europe, using sequences of the mitochondrial 16S rDNA gene. The ML (Maximum Likelihood) tree revealed two clades within Physidae, and two clades that correspond to the families Planorbidae and Lymnaeidae. In the Physidae clade there are two separate clades: one includes the species *Physa spelunca*, and the second includes samples of *P. acuta*. We determined three different haplotypes within specimens from Serbia. One haplotype is genetically closest to *Physa heterostrofa* (synonym of *P. acuta*) from Philadelphia, while the other two are very close to *P. acuta* specimens from New Mexico. Together with other samples, our findings corroborate the notion that we are dealing with one panmictic population of *P. acuta* and not with several separate species, despite the high genetic diversity between and among the populations. Our results indicate that in the same population in Serbia, there is high genetic distance between samples. Despite the small number of analyzed samples, our findings point to multiple introductions of *P. acuta* from different locations in America.

Keywords: 16S rDNA variation, Physidae, *Physa acuta*, alien species, Danube, Serbia.

Introduction

Physa (= *Physella*) are the most abundant and diverse freshwater gastropods native to North America, which due to their invasive nature occur throughout the world (Burch, 1989). They are hermaphrodites with the ability to self-fertilize and can be found in a wide variety of freshwater habitats worldwide. Dillon (2000) provides an excellent ecological overview of this group. Based primarily on shell characteristics over 30 species have been described (Te, 1980; Burch, 1989).

The Physidae family can be divided into six groups, i.e. *aplexa*, *marmorata*, *fontinalis*, *acuta*, *gyrina* and *pomili*, which are phylogenetically different, possess distinctive penial morphology, and to some extent distinctive shell morphology (Te, 1978; Wethington, 2004). The influence of environmental factors and genetic variability must also be taken into consideration when distinguishing members of the family, since the use of only shell morphology can lead to misidentification (DeWitt *et al*., 2000). Taylor's (2003) classification scheme of the Physidae, was primarily based on the penial complex. The author assumed that changes in penial morphology were progressive, and classified the family into grades and clades based on whether groups possess primitive or specialized characteristics.

Members of the *acuta* group can be identified by the following characters: shell shape is oval and the spire is pointed (acute); the aperture is large and narrow at the top; the shell is dull and striated longitudinally (DeWitt *et al*., 2000). This group is characterized by considerable genetic variation, both within species and within populations. This variation, along with the absence of reproductive isolation between nominal species, has led to many species within this group to be lumped together under the name *Physa acuta*, including *P. heterostropha*, *P. integra*, *P. cubensis*, and *P. virgata* (Dillon *et al*., 2002; Wethington 2004). Taylor (2003) has transferred *Physa* (*Physella) acuta* to the genus *Haitia* Clench & Aguayo, 1932, but herein we maintain the name *P. acuta*, following the classification of Wethington (2004).

Physa acuta was recorded in most parts of Europe, up to Sweden to the north, the British Isles to the west, Ukraine to the east, and the Mediterranean to the south. It has been also introduced to Africa, Asia and Australia by human commerce (de Jong *et al*., 2014). In Serbia, according to Novaković (2014), *P. acuta* was recorded in a total of 74 sampling sites situated at 52 watercourses and 4 reservoirs. In Vojvodina, the species is present mainly in the Danube–Tisa–Danube Canal (DTD), and in small and medium watercourses at altitudes up to 500 m (Novaković, 2014).

The aim of this study was to explore the genetic diversity of *P. acuta* from Serbia, and to determine the evolutionary relationships of the examined population with native Physidae populations from the United States of America, Canada, Mexico and Cuba, as well as with populations from Europe, using mitochondrial 16S rDNA gene sequences.

Materials and Methods

Fieldwork and species identification

Investigation of freshwater mollusks was performed during sampling of the faunal composition of the wetlands (backwater) in the Danube River (Fig. 1). The samples were collected along the right bank of the river at the following localities, the Čibuklija wetland (44.804719° N, 21.319505° W; river km 1082.70) and along the left bank of the river, the Žilova wetland (44.778011° N, 21.191754° W; river km 1090.50). Fieldwork was conducted in 2010, 2011, and 2014, using the Van-Veen type of grab with a sample area of 270 cm² and a "kick-netting" type of benthos network (mesh size 500 µm). After collection, samples were transferred to the laboratory where species were identified under a stereomicroscope (MBL2000, Krüss, Germany) and a binocular magnifier (MSZ5000, Krüss, Germany) according to Zhadin (1952), Pfleger (1999), Glöer (2002) and Glöer & Meier-Brook (2003). Table 1 provides a summary of the sampling locations, the number of individuals typed for the mitochondrial large subunit rDNA (16S) marker, and voucher specimen information for the Serbian *P. acuta* and sequences taken from GenBank database at the NCBI (http://www.ncbi.nlm.nih.gov/, National Center for Biotechnology Information). Additionally, seven specimens were included as representative of other freshwater Basommatophora families (Lymnaeidae, Planorbidae; see Table 1) as related species from the same habitat.

Isolation of soft tissue from snails

Individuals used for the molecular analyses were transferred to the laboratory alive, without use of a fixative. The soft tissues were isolated using the microwave oven (MWO) method, by treating individual specimens with microwaves in a microwave oven MO17DE, Gorenje (output power of 700 W) at various intervals, depending on the length of the shell, and according to a previously published protocol (Galindo *et al.*, 2014), modified for freshwater snails (see Appendix I). The MWO method allowed for the preservation of undamaged snail shells that have been deposited in the collection of the Department of Hydroecology and Water Protection, Institute for Biological Sciences "Siniša Stanković", University of Belgrade, Serbia. For molecular analyses, the soft parts of the body were stored in 96% ethanol.

DNA isolation, amplification and sequencing

DNA was extracted using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. For larger specimens, 20-30 mg of foot tissue was used. The target gene fragment was amplified using the following primers: 16Sar '5-CGCCTGTTTATCAAAAACAT-3' and 16Sbr '5-CCGGTCTGAACTCAGATCACGT-3' (Kessing *et al.*, 1989). DNA amplification was performed in a final volume of 25 ml, containing 1 ml of the extracted DNA, 12.5 ml of 1 x KAPA2G Robust HotStart ReadyMix (containing 2 mM $MgCl_2$ and 1.25 ml of 0.5 µM of each primer, and 9 ml of water. PCR was performed in an Eppendorf Mastercycler® (Hamburg, Germany) using the following thermal profile: initial denaturation at 94°C for 3 min, 38 cycles at 94°C for 30 s, at 56°C for 30 s, at 72°C for 45 s, and a final extension step at 72°C for 7 min. The PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. DNA sequencing in both directions was performed by Macrogen Europe Inc. (Amsterdam, Netherland).

Phylogenetic analysis

For the detection of defects and polymorphic sites at the ends of the sequences we used Sequencher 5.2.4. software (Trial free version). Comparison of the obtained sequences with sequences in the gene bank database was performed using the Basic Local Alignment Tool (BLAST), available at http://www.ncbi.nlm.nih.gov. Sequences were aligned using the program Clustal_W with the parameters provided in the software package MEGA (version 6.0; Tamura *et al.*, 2013). For calculation of average genetic distances, Kimura's two-parameter method (K2P) of base substitution was used. The Maximum Likelihood (ML) tree was obtained using MEGA 6 software. The robustness of the tree was assessed using a bootstrap analysis with 1.000 replicates. The haplotype network was constructed with the TCS program (version 1.21; Clement *et al*., 2000).

Results

P. acuta was found in the Čibuklija and Žilova wetlands of the Danube. Beside *P. acuta*, we recorded several other species from the order Basommatophora,

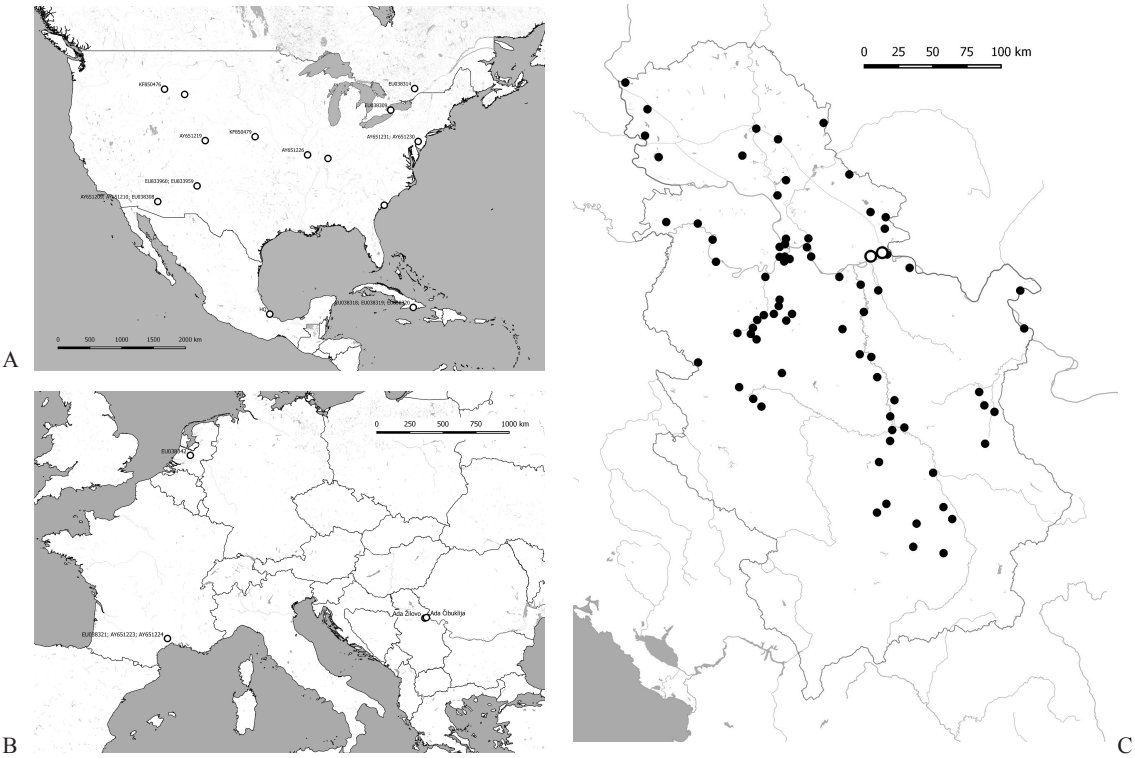

Fig. 1: Distribution of *P. acuta* group in Canada, North America, Mexico and Cuba (A), Europe (B) and Serbia (C). Distribution of *P. acuta* in North and Central America (white circles) was compiled from the data of Wethington *et al*. (2009), Wethington & Guralnick (2004) and Kraus *et al*. (2014). The European (white circles) range is based on Wethington & Lydeard (2007), data and distribution in Serbia is based on Novaković (2014, black circles), and on our field sampling data (white circles).

which were collected for molecular analysis. In total, six specimens of *P. acuta* and *Planorbarius corneus* and one specimen of *Lymnaea stagnalis* and *Stagnicola palustris* (Table 1) were collected. All collected specimens of *P. acuta* were treated as a single population, since both wetlands / marshes are a few kilometers apart and their habitat features are the same.

The ML tree inferred with the alignment of the matrix, including 51 specimens, revealed two clades within Physidae, and two clades corresponding to the families Planorbidae and Lymnaeidae (Fig. 2). Within the clade Physidae there are two separate clades: one contains the species *P. spelunca* (acuta group B), and the second includes the rest of the samples of *P. acuta* (acuta group A). We identified three different haplotypes in the specimens from Serbia. One haplotype was genetically closest to the species *P. heterostrofa* (now considered as synonym of *P. acuta*) from Philadelphia, while two others were very close to *P. acuta* specimens from New Mexico.

Haplotype networks of *P. acuta* samples, together with *P. spelunca* (Fig. 3) revealed that samples from Europe possess distant haplotypes in the network matrix. These results confirmed that in Serbia, in the same population, there is a high genetic variability between samples. K2P distances varied between our samples from 3.5 to 3.7%. Moreover, samples from France, similarly to the Serbian samples, are clearly separated in two different clusters, with K2P distances of 2.8-3.5% (See Appendix II).

Discussion

Biodiversity of freshwater ecosystems is changing rapidly due to anthropogenic transoceanic biotic exchange, and water habitats are considerably affected by biological invasions (Pyšek *et al*., 2010), as has already been described for the river Danube (Paunović *et al*., 2007a,b; Arbaciauskas *et al*., 2008; Sommerwerk *et al*., 2009; Sommerwerk *et al*., 2010; Paunović *et al*., 2015). Invasive species can be introduced by intent or by chance. Introduced species compete with native inhabitants for resources, which can cause suppression of native species from their natural habitats. With regard to Serbia, it has been confirmed that the rate of introduction of alien species in some biotopes is growing, as they are suitable recipient areas (Zorić *et al*., 2010a). For the largest number of alien species, the most suitable recipient areas are heavily modified and anthropo-

Table 1. Locality details, GenBank accession numbers and status of the freshwater snails typed for mt16S region. I = introduced (Europe); N = native (America).

Taxon	Locality	16S (N/hN)*	Specimen code	Status	GenBank Accession#
Physidae					
Physa acuta	France; Montpellier	1/1		I	AY651224
Physa acuta	France; Montpellier	1/1		I	AY651223
Physa acuta	France; Montpellier	1/1		I	EU038321
Physa acuta	Netherlands	1/1		I	EU038342
Physa acuta	Serbia; Danube	3/1	mr21; mr23; mr24	I	
Physa acuta	Serbia; Danube	2/1	mr19; mr20	I	
Physa acuta	Serbia; Danube	1/1	mr22	I	
Physa acuta	Nebraska	1/1		N	KF850479
Physa acuta	Montana	1/1		N	KF850476
Physa acuta	Wyoming	1/1		N	AY651241
Physa acuta	Missouri	1/1		N	AY651226
Physa acuta	Arizona	1/1		N	EU038308
Physa acuta	Niagara River, Canada	1/1		N	EU038309
Physa acuta	Colorado	1/1		N	AY651219
Physa acuta	Charleston; South Carolina	2/1		N	GQ415017; GQ415009
Physa acuta	Charleston; South Carolina	1/1		N	GQ415021
Physa acuta	Charleston; South Carolina	1/1		N	GQ415010
Physa acuta	Charleston; South Carolina	1/1		N	GQ415020
Physa acuta	Charleston; South Carolina	1/1		N	GQ415019
Physa acuta	Charleston; South Carolina	1/1		N	GQ415018
Physa acuta	Charleston; South Carolina	1/1		N	GQ415016
Physa acuta	Charleston; South Carolina	1/1		N	GQ415015
Physa acuta	Charleston; South Carolina	1/1		N	GQ415014
Physa acuta	Charleston; South Carolina	1/1		N	GQ415013
Physa acuta	Charleston; South Carolina	1/1		N	GQ415012
Physa acuta	New Mexico	1/1		N	EU833960
Physa acuta	New Mexico	1/1		N	EU833959
Physa acuta	New Harmony, Indiana	1/1		N	EU038325
Physa acuta	New Harmony, Indiana	1/1		N	EU038324
Physa acuta	Cuba	1/1		N	EU038319
Physa acuta	Cuba	2/1		N	EU038320; EU038318
Physa acuta	Mexico	1/1		I	HQ283247
Physa heterostropha	Pennsylvania, Philadelphia	1/1		N	AY651231
Physa heterostropha	Pennsylvania, Philadelphia	1/1		N	AY651230
Physa spelunca	Lower Kane Cave; Wyoming	1/1		N	AY651243
Physa spelunca	Lower Kane Cave; Wyoming	1/1		N	AY651242
Physa virgata	Gila River; Arizona	1/1		N	AY651210
Physa virgata	Gila River; Arizona	1/1		N	AY651209
Lymnaeidae					
Lymnaea stagnalis	Serbia; Danube	1/1	mr33	N	
Stagnicola palustris	Serbia; Danube	1/1	mr18	N	
Planorbidae					
Planorbarius corneus	Serbia; Danube	2/1	mr26; mr31	N	
Planorbarius corneus	Serbia; Danube	1/1	mr27	N	
Planorbarius corneus	Serbia; Danube	1/1	mr28	N	
Planorbarius corneus	Serbia; Danube	1/1	mr29	N	
Planorbarius corneus	Serbia; Danube	1/1	mr32	N	

* N = number of individuals sequenced; hN = number of unique haplotypes obtained.

genic aquatic habitats, which are also suitable for the spread of the alien populations of *P. acuta*. Moderate to high level of organic pollution, intensive inland water navigation and invasive species corridors in the vicinity also contribute to the degree of successful invasiveness of an area. In Europe, there are four main invasive corridors: the northern, central, western and southern corridors. The southern corridor links the Black and North Sea by the Danube, Rhine and Main rivers and is the most important corridor for the spread of alien species (Bij de Vaate *et al*., 2002; Panov *et al*., 2009; Panov *et al*., 2010).

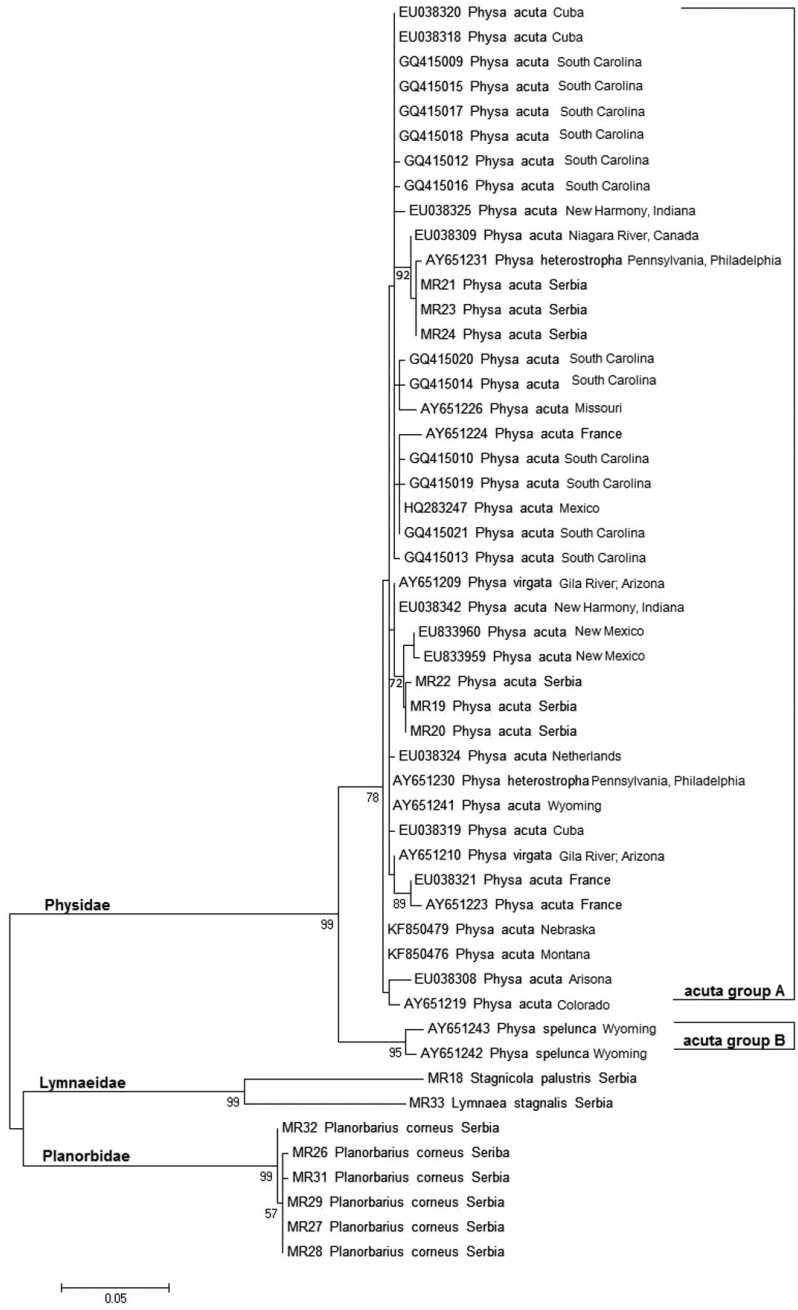

Fig. 2: Phylogenetic trees based on mt16S rDNA, obtained using the Maximum Likelihood (ML) method. Bootstrap values are indicated below the branches. Scale bar indicates the number of substitutions per site.

The Danube is one of the most interesting routes of introduction and spread of aquatic alien species, with the spreading continuing through its main tributaries, the Sava, the Tisa and the Morava. This corridor was most probably the main corridor through which *P. acuta* spread to Serbia. That is also supported by the high number of individuals recorded at the Sava River during the "GLOBAQUA" project surveys of 2014 and 2015 (unpublished data). We believe that wetland birds contribute to the spread of North American species along the Danube, as Malone (1965) linked the spread of North American species into Europe with wetland birds. The snail *P.*

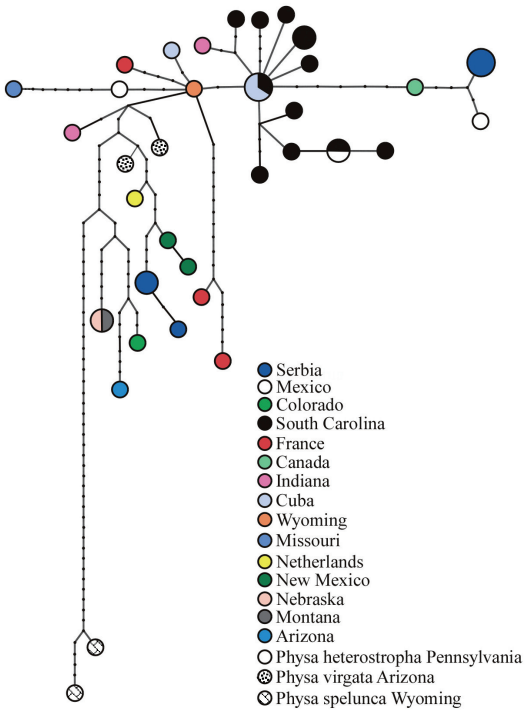

Fig. 3: Haplotype networks from 43 *Physa acuta* group specimens, obtained using statistical parsimony (TCS). Circles represent specific haplotypes; the size of the circles reflects the number of individuals with a particular haplotype (not to scale); the dots between the circles represent mutational steps.

acuta has now attained worldwide distribution and can be considered as one of the most cosmopolitan recent snail species (Dillon *et al*., 2002; Wethington & Lydeard, 2007). This species is known as a rapid (re)coloniser of freshwater systems of variable environmental conditions (Chlyeh *et al*., 2006). Overall, in Serbian rivers, 21 alien species of aquatic macroinvertebrates have been identified and classified as white, black and grey, depending on their degree of invasiveness (Zorić *et al.*, 2010a,b; Raković, 2015).

The family Physidae is characterized by high intraspecies genetic variability (Journey, 2014), which is corroborated byour results. The first clade, which includes *P. spelunca,* was observed to be phylogenetically distinct, as expected. This species lives in a hit spring in a cave, filled with toxic sulphuric gas and feeds primarily on bacteria (Turner & Clench, 1974). Differences in penial morphology of the acuta group have been observed, i.e. the eastern forms (*P. acuta*, *P. heterostropha* and *P. integra*) have transparent, muscular penial sheaths, while the sheaths of the western forms (*P. virgata* and *P. spelunca*) are less transparent and more opaque (Wethington & Lydeard, 2007).

The second clade within the Physidae is characterized by relatively high genetic distance (up to 5.58%) for the analyzed 16S rDNA gene, and this result is consistent with previous findings, of samples from other regions (Wethington *et al*., 2009). The samples from Serbia exhibit considerably distant positions in the haplotype network of the *P. acuta* group. One haplotype group clusters with samples from Canada and Pennsylvania, while the other group is closer to a population from New Mexico, corroborating previous claims that these are actually one panmictic population and not a separate species. The high genetic variability, determined in both the native and the introduced populations, makes it difficult to draw conclusions about the origin of the invasive stock in Europe and Serbia. In addition, despite the high genetic differences within *P. acuta*, there is no reproductive isolation, as reported by Dillon *et al.* (2002), who sampled six populations from diverse environments across North America and Europe. These authors also suggested that the North American populations appear to be relatively stable and morphologically diverse; their range is largely prehistoric and taxonomists have recognized numerous subspecies and forms. Elsewhere the species seems to be more morphologically uniform and biologically invasive, which is the case of the population in Serbia. The high genetic variability of alien populations of *P. acuta* most likely contributes to the success of this species in its establishment, maintenance and dispersal in new habitats (Hartl & Clark, 1989). The observed high genetic variability, despite the small number of analyzed samples, also suggests that there were potentially multiple introductions of the species from different locations. Further studies are required to further elucidate the dynamics of *P. acuta*.

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Appendix I

A modified protocol for freshwater snails using MWO method:

I: Small snails – snails with shell length of 2-5 mm they were treated with electromagnetic waves in the microwave oven for 5-9 s: with 2 mm - 5 s / *Gyraulus crista* (Linnaeus, 1758); 4 mm - 6 s / *Bathyomphalus contortus* (L., 1758); 4 mm - 7 s / *Segmentina nitida* (M. 1774);

II: Medium snails – snails with shell length of 5-20 mm were treated with electromagnetic waves in the microwave oven for 8-20 s: with 8 mm - 10 s / *Anisus vortex* (L., 1758); 9 mm - 12 s (13 mm - 15 s) / *Physa acuta* (D. 1805); 15 mm - 17 s (18 mm - 20 s) / *Planorbis* (L., 1758); 19 mm - 20 s / *Radix labiata* (R. 1835);

III: Large snails – snails with shell length of 20-40 mm were treated with electromagnetic waves in the microwave oven for a period of 20-40 s: with 25 mm - 30 s (35 mm - 40 s) / *Planorbarius corneus* (L., 1758);

IV: The largest snails – snails with shell length greater than 40 mm were treated with electromagnetic waves in the microwave for over 40 s: 47 mm - 50 s / *Lymnaea stagnalis* (L., 1758).

Appendix II

Estimates of evolutionary divergence between sequences P. acuta group.

	Locality*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
1	Nebraska																																										
2	Pennsylvania	0.026																																									
3	Pennsylvania	0.011	0.028																																								
4	Montana	0.000	0.026	0.011																																							
5	Arizona	0.007	0.028	0.013	0.007																																						
6	Arizona	0.013	0.030	0.015	0.013	0.015																																				
7	Mexico	0.011	0.022	0.013	0.011	0.013	0.015																																			
8	South Carolina	0.011	0.022	0.013	0.011	0.013	0.015	0.000																																		
9	South Carolina	0.013	0.024	0.015	0.013	0.015	0.017	0.007	0.007																																	
10	South Carolina	0.013	0.024	0.015	0.013	0.015	0.017	0.002	0.002	0.004																																
11	South Carolina	0.009	0.020	0.011	0.009	0.011	0.013	0.002	0.002	0.004	0.004																															
12	South Carolina	0.009	0.020	0.011	0.009	0.011	0.013	0.002	0.002	0.004	0.004	0.000																														
13	South Carolina	0.011	0.022	0.013	0.011	0.013	0.015	0.004	0.004	0.007	0.007	0.002	0.002																													
14	Wyoming	0.069	0.080	0.072	0.069	0.076	0.074	0.072	0.072	0.074	0.074	0.069	0.069	0.072																												
15	Wyoming	0.069	0.085	0.072	0.069	0.076	0.069	0.076	0.076	0.078	0.078	0.074	0.074	0.076	0.013																											
16	South Carolina	0.009	0.020	0.011	0.009	0.011	0.013	0.002	0.002	0.004	0.004	0.000	0.000	0.002	0.069	0.074																										
17	South Carolina	0.013	0.024	0.015	0.013	0.015	0.017	0.007	0.007	0.004	0.009	0.004	0.004	0.007	0.074	0.078	0.004																									
18	South Carolina	0.013	0.024	0.015	0.013	0.015	0.011	0.007	0.007	0.009	0.009	0.004	0.004	0.007	0.072	0.076	0.004	0.009																								
19	South Carolina	0.011	0.022	0.013	0.011	0.013	0.015	0.004	0.004	0.007	0.007	0.002	0.002	0.004	0.072	0.076	0.002	0.007	0.007																							
20	South Carolina	0.013	0.024	0.015	0.013	0.015	0.017	0.002	0.002	0.004	0.004	0.004	0.004	0.007	0.074	0.078	0.004	0.004	0.009	0.007																						
21	South Carolina	0.009	0.020	0.011	0.009	0.011	0.013	0.002	0.002	0.004	0.004	0.000	0.000	0.002	0.069	0.074	0.000	0.004	0.004	0.002	0.004																					
22	New Mexico	0.017	0.035	0.020	0.017	0.020	0.013	0.020	0.020	0.020	0.022	0.017	0.017	0.020	0.080	0.076	0.017	0.020	0.017	0.020	0.020	0.017																				
23	New Mexico	0.020	0.037	0.022	0.020	0.022	0.015	0.022	0.022	0.022	0.024	0.020	0.020	0.022	0.082	0.078	0.020	0.022	0.020	0.022	0.022	0.020	0.002																		
24	Netherlands	0.015	0.033	0.017	0.015	0.017	0.009	0.017	0.017	0.020	0.020	0.015	0.015	0.017	0.076	0.072	0.015	0.020	0.015	0.017	0.020	0.015	0.011	0.013																		
25	Indiana	0.013	0.024	0.015	0.013	0.015	0.017	0.007	0.007	0.009	0.009	0.004	0.004	0.007	0.074	0.078	0.004	0.009	0.009	0.007	0.009	0.004	0.022	0.024	0.020																	
26	Indiana	0.009	0.026	0.011	0.009	0.011	0.013	0.011	0.011	0.009	0.009	0.009	0.009	0.011	0.074	0.074	0.009	0.013	0.013	0.011	0.013	0.009	0.017	0.020	0.015	0.013																
27	Serbia	0.022	0.039	0.024	0.022	0.024	0.017	0.024	0.024	0.022	0.026	0.022	0.022	0.024	0.085	0.080	0.022	0.022	0.022	0.024	0.022	0.022	0.011	0.013	0.015	0.026	0.022															
28	Serbia	0.022	0.039	0.024	0.022	0.024	0.017	0.024	0.024	0.022	0.026	0.022	0.022	0.024	0.085	0.080	0.022	0.022	0.022	0.024	0.022	0.022	0.011	0.013	0.015	0.026	0.022	0.000													
29	Serbia	0.022	0.004	0.024	0.022	0.024	0.026	0.017	0.017	0.020	0.020	0.015	0.015	0.017	0.078	0.082	0.015	0.020	0.020	0.017	0.020	0.015	0.030	0.033	0.028	0.020	0.022	0.035	0.035												
30	Serbia	0.024	0.041	0.026	0.024	0.026	0.020	0.026	0.026	0.024	0.028	0.024	0.024	0.026	0.087	0.082	0.024	0.024	0.024	0.026	0.024	0.024	0.013	0.015	0.017	0.028	0.024	0.002	0.002	0.037											
31	Serbia	0.022	0.004	0.024	0.022	0.024	0.026	0.017	0.017	0.020	0.020	0.015	0.015	0.017	0.078	0.082	0.015	0.020	0.020	0.017	0.020	0.015	0.030	0.033	0.028	0.020	0.022	0.035	0.035	0.000	0.037										
32	Serbia	0.022	0.004	0.024	0.022	0.024	0.026	0.017	0.017	0.020	0.020	0.015	0.015	0.017	0.078	0.082	0.015	0.020	0.020	0.017	0.020	0.015	0.030	0.033	0.028	0.020	0.022	0.035	0.035	0.000	0.037	0.000									
33	France	0.024	0.039	0.024	0.024	0.022	0.024	0.026	0.026	0.028	0.028	0.024	0.024	0.026	0.082	0.082	0.024	0.028	0.028	0.026	0.028	0.024	0.033	0.035	0.028	0.028	0.024	0.037	0.037	0.035	0.039	0.035	0.035								
34	Cuba	0.009	0.020	0.011	0.009	0.011	0.013	0.002	0.002	0.004	0.004	0.000	0.000	0.002	0.069	0.074	0.000	0.004	0.004	0.002	0.004	0.000	0.017	0.020	0.015	0.004	0.009	0.022	0.022	0.015	0.024	0.015	0.015	0.024							
35	Cuba	0.009	0.026	0.011	0.009	0.011	0.013	0.011	0.011	0.013	0.013	0.009	0.009	0.011	0.074	0.074	0.009	0.013	0.013	0.011	0.013	0.009	0.017	0.020	0.015	0.013	0.009	0.022	0.022	0.022	0.024	0.022	0.022	0.022	0.009						
36	Cuba	0.009	0.020	0.011	0.009	0.011	0.013	0.002	0.002	0.004	0.004	0.000	0.000	0.002	0.069	0.074	0.000	0.004	0.004	0.002	0.004	0.000	0.017	0.020	0.015	0.004	0.009	0.022	0.022	0.015	0.024	0.015	0.015	0.024	0.000	0.009					
37	Canada	0.017	0.009	0.020	0.017	0.020	0.022	0.013	0.013	0.015	0.015	0.011	0.011	0.013	0.074	0.078	0.011	0.015	0.015	0.013	0.015	0.011	0.026	0.028	0.024	0.015	0.017	0.030	0.030	0.004	0.033	0.004	0.004	0.030	0.011	0.017	0.011				
38	Arizona	0.024	0.046	0.028	0.024	0.030	0.030	0.030	0.030	0.033	0.033	0.028	0.028	0.030	0.067	0.067	0.028	0.033	0.033	0.030	0.033	0.028	0.037	0.039	0.033	0.033	0.028	0.039	0.039	0.041	0.041	0.041	0.041	0.037	0.028	0.028	0.028	0.037			
39	Wyoming	0.004	0.022	0.007	0.004	0.007	0.009	0.007	0.007	0.009	0.009	0.004	0.004	0.007	0.069	0.069	0.004	0.009	0.009	0.007	0.009	0.004	0.013	0.015	0.011	0.009	0.004	0.017	0.017	0.017	0.020	0.017	0.017	0.020	0.004	0.004	0.004	0.013	0.024		
40	Missouri	0.024	0.039	0.013	0.024	0.026	0.028	0.024	0.024	0.022	0.026	0.022	0.022	0.024	0.082	0.085	0.022	0.022	0.026	0.024	0.022	0.022	0.030	0.033	0.030	0.026	0.024	0.028	0.028	0.035	0.030	0.035	0.035	0.037	0.022	0.024	0.022	0.030	0.041	0.020	
41	France	0.013	0.030	0.015	0.013	0.015	0.017	0.011	0.011	0.017	0.013	0.013	0.013	0.015	0.078	0.078	0.013	0.017	0.017	0.015	0.013	0.013	0.022	0.024	0.020	0.017	0.013	0.026	0.026	0.026	0.028	0.026	0.026	0.028	0.013	0.013	0.013	0.022	0.033	0.009	0.028
42	France	0.030	0.046	0.030	0.030	0.028	0.028	0.033	0.033	0.030	0.030	0.030	0.030	0.033	0.089	0.087	0.030	0.035	0.035	0.033	0.035	0.030	0.037	0.039	0.033	0.035	0.026	0.041	0.041	0.041	0.039	0.041	0.041	0.007	0.030	0.028	0.030	0.037	0.043	0.026	0.043	0.035
43	Colorado	0.015	0.037	0.022	0.015	0.022	0.022	0.022	0.022	0.024	0.024	0.020	0.020	0.022	0.067	0.067	0.020	0.024	0.024	0.022	0.024	0.020	0.028	0.026	0.024	0.024	0.020	0.030	0.030	0.033	0.033	0.033	0.033	0.033	0.020	0.020	0.020	0.028	0.017	0.015	0.035	0.024	0.039

* Locality of Physa acuta group specimens.