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Morphometric variations among *Astacus astacus* populations from different regions of the Balkan Peninsula

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Abstract The morphometric variation among several populations of *Astacus astacus* from different regions of the Balkan Peninsula was studied based on a large set of morphometric parameters per crayfish. Differences in morphometry among populations from ten water bodies in Serbia, Slovenia and Albania were tested using a multiple discriminant analysis. Analyses included twenty-two morphometric parameters per crayfish. The most discriminant characteristics for separating males among populations were weight, head width (HEW), width of the carapace at the hind edges (CEW) and claw length (CLL), and for female populations those were abdomen length (ABL), rostrum width (ROW), total length (TL), claw length (CLL) and claw width (CLW). Our results improve the existing knowledge about the noble crayfish morphology, show clear differentiation between populations from various aquatic ecosystems and reflect geographical separation.

Keywords Noble crayfish · Morphometric characteristics · Multivariate statistics · Serbia · Slovenia · Albania

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Introduction

Freshwater ecosystems are characterized by a high spatial complexity, and this heterogeneity affects the distribution of aquatic organisms in riverine landscapes (Hepp et al. 2012). Freshwater crayfish are known as important components of biodiversity in rivers, lakes and wetlands, with crucial ecological role in the appropriate freshwater ecosystem functioning (Holdich 2002; Gherardi 2011), and with an important role in the food webs (Nyström et al. 1996; Usio and Townsend 2004; Sint et al. 2007; Zimmerman 2012). Europe is natively populated by five indigenous crayfish species whose abundance and distribution have been greatly altered and reduced due to climate changes, pollution, habitat degradation, introduction of alien crayfish species and crayfish plague (disease caused by the Oomycete *Aphanomyces astaci* (Schikora, 1906)) (Fevolden and Hessen 1989; Füreder et al. 2006; Souty-Grosset et al. 2006; Holdich et al. 2009; Papavlasopoulou et al. 2014). Also, the natural genetic structure of the species has been greatly impaired, mixed and diminished in large parts of Europe due to the high amount of manmade crayfish translocations and stockings (Grandjean and Souty-Grosset 2000; Schrimpf et al. 2011, 2014; Gross et al. 2013; Makkonen et al. 2015).

One of the native freshwater crayfish species, once widely distributed throughout Europe, including the Balkan Peninsula, is the noble crayfish, *Astacus astacus* (Linnaeus, 1758) (Kouba et al. 2014). Its native range extends from Russia and Ukraine in the east, to the UK and France in the west, to Finland, Sweden and Norway in the north and Greece in the south (Edsman et al. 2010). During the last century, many populations of this species diminished or became extinct (Holdich et al. 2009; Edsman et al. 2010; Schrimpf et al. 2011). Therefore, the noble crayfish is listed as "vulnerable" on the IUCN (International Union for the Conservation of Nature and Natural Resources) Red List of Threatened Species (IUCN 2015; Edsman et al. 2010). In Slovenia, the noble crayfish, along with two other indigenous crayfish species (stone and white-clawed crayfish), and their habitats are protected by national law (Anonymous 2004). They are listed as "vulnerable" on the national Red list of the Species (Anonymous 2002) and are also included in the national monitoring program. In Serbia, this species is classified as "endangered" and is strictly protected species (Anonymous 2010a, b). Morphometric measurements analyzed by multivariate statistical methods offer a good tool to explore differences between single groups of organisms. Moreover, analysis of crayfish morphometric features can be used for the distinction of populations without being as expensive as genetic analysis (Sint et al. 2005; Maguire and Dakić 2011). Traditional multivariate morphometrics, accounting for variation in size and shape, have successfully discriminated many fish stocks (Cadrin and Friedland 1999) as well as crayfish (Sint et al. 2005, 2007; Maguire and Dakić 2011). The first studies on the noble crayfish biogeography and taxonomy were based on morphological characteristics (Karaman M. S. 1962, 1963; Albrecht 1983).

However, morphometric characteristics of the noble crayfish populations from Serbia, Slovenia and Albania have not yet been investigated. Previous work on the crayfish in Serbia included studies on their distribution (Karaman S. 1929; Karaman M. S. 1962, 1963; Simić et al. 2008), population status, degree of threat (Simić et al. 2008) and conservation strategy (Simić et al. 2015). In Slovenia, the previous crayfish studies were related mainly to the invasive species and their establishment (Jaklič and Vrezec 2011), to the crayfish distribution (Machino 1997; Bedjanič 2004), their physiological response (Simčič et al. 2012a, b, 2014), phylogeny and phylogeography (Trontelj et al. 2005) and presence of *Aphanomyces astaci* (Kušar et al. 2013). Studies in Albania included only the occurrence of epibiotic Branchiobdellida species on the crayfish (Subchev 2011), where some information on the host crayfish species was made available.

The aims of this study were (1) to determine whether populations of the noble crayfish from distant regions of the Balkan Peninsula are morphologically different and (2) to contribute to the knowledge on the morphology of the noble crayfish from this area as the basis for the forthcoming genetic research.

Materials and methods

We conducted the field work and specimen collection in the three countries (Serbia, Slovenia, and Albania) from the Balkan Peninsula (Fig. 1). Altogether ten populations of *A. astacus* were sampled, with permission of local authorities, seven from Serbia, two from Slovenia and one from Albania. The number of individuals per population is presented in Table 1.

In Serbia, crayfish were sampled by hand, in shallow streams and rivers, or trapped with baited LiNi traps (Westman et al. 1978) that were left in bigger rivers and reservoirs overnights. We used fresh chicken or pork liver as bait. In Slovenia, classical cylindrical crayfish traps (76 × 23 cm) with funnel entrances on both ends were used for sampling, with fresh pork liver as bait (Holdich 2002). The traps were set into the water during the afternoon and collected the following morning. Albanian crayfish were purchased from a fisherman on Lake Prespa. All crayfish were sexed, and only unharmed specimens, without signs of regeneration, were measured according to Sint et al. (2005). Each specimen was also weighted to the nearest 0.01 g with electrical balance. Body mass was expressed as wet weight (WW) in grams (g). After measurement, crayfish were released at the place where they were caught.

We measured 21 morphometric characteristic, adopted from Sint et al. (2005) with a Vernier caliper to the nearest 1.0 mm for each specimen: total length (TL), rostrum length (ROL), rostrum width (ROW), head length (HEL), head width (HEW), areolar length (ARL), areolar width (ARW), abdomen length (ABL), abdomen width (ABW), abdomen height (ABH), telson length (TEL) and telson width (TEW), carapace width (CPW), width at the cervical groove (CGW), width of the carapace at the hind edges (CEW), carapace height (CPH), claw length (CLL), claw width (CLW), claw height (CLH), length of the claw palm (CPL) and length of the claw finger (CFL). The specimens whose total length was less than 70 mm were excluded from analysis in order to avoid immature individuals (Sint et al. 2007).

All of the measured characteristics were normalized for size by dividing them with the corresponding postorbital length (POL = HEL + ARL) (Sint et al. 2005; Maguire and Dakić 2011).

Statistics

Analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, USA) with significance level set at $p < 0.05$. Parametric tests (*t* test, ANOVA with post hoc Bonferroni test) were used when the data met assumptions for such tests (i.e., normality/homoscedasticity) (Zar 1999). Morphometric differentiation among *A. astacus* populations in the Balkan Peninsula was examined and evaluated by analyzing all of the measured variables simultaneously with discriminant functional analysis.

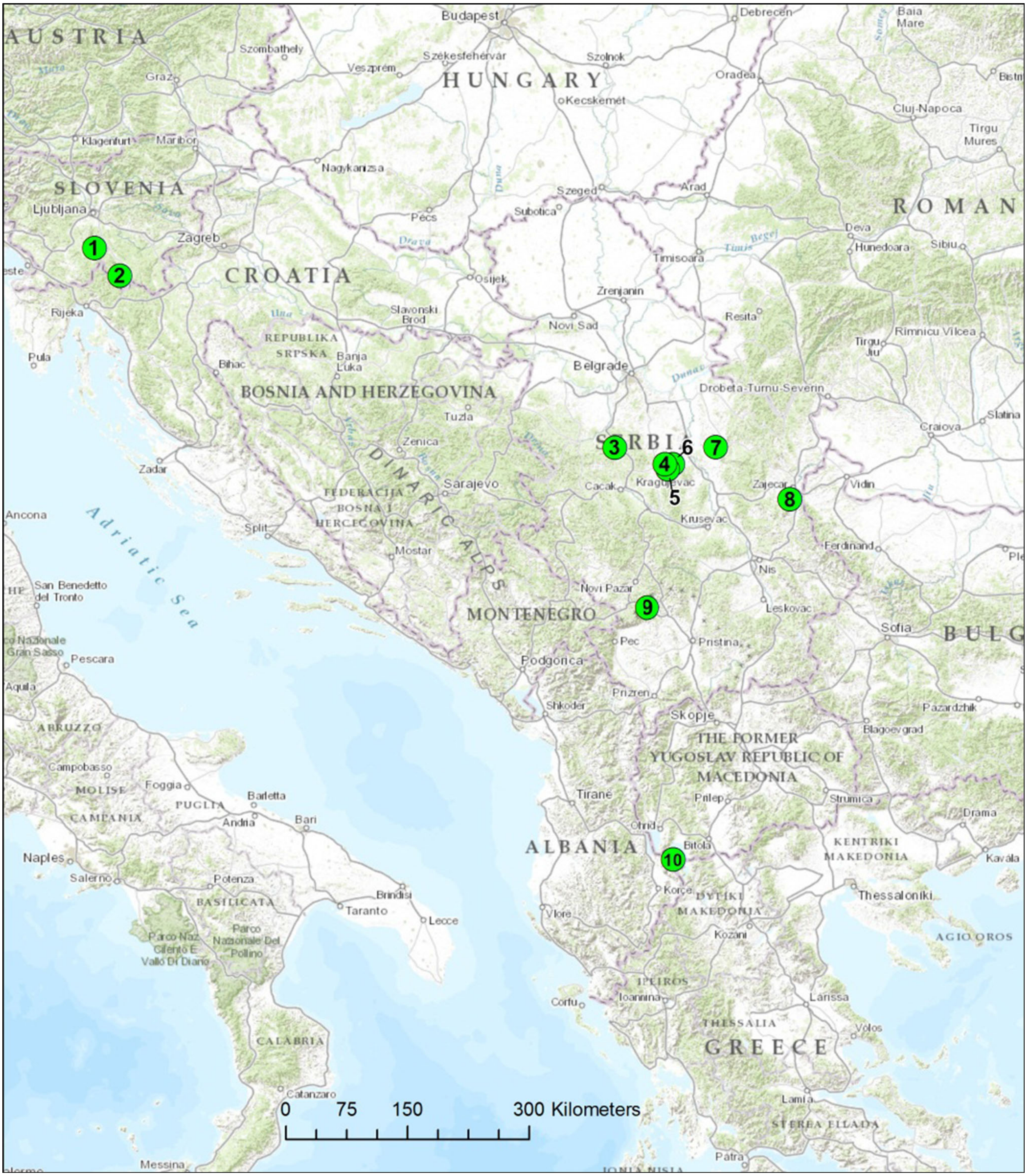

Fig. 1 Map of sampling location: *1* the Bloščica River; *2* the Kočevska River; *3* the Kačer River; *4* the Milošev fishpond; *5* the Petrovačka River; *6* the Resnički Stream; *7* the Korenica Reservoir; *8* the Grliško Reservoir; *9* the Gazivode Reservoir; *10* Lake Prespa

Table 1 Geographical coordinates of studied locations and number of specimens [females (N_f) and males (N_m)] per population

Locations/populations	WGS84 coordinates		Number of specimens		
	N	*E*	N_f	N_m	Total
Resnički Stream, SRB	44.090175	20.937267	58	34	92
Petrovačka River, SRB	44.052394	20.877971	10	23	33
Kačer River, SRB	44.221652	20.280370	3	15	18
Korenica Reservoir, SRB	44.227491	21.413162	8	5	13
Grliško Reservoir, SRB	43.811844	22.231652	6	9	15
Gazivode Reservoir, SRB	42.941469	20.647488	8	16	24
Milošev fishpond, SRB	44.091776	20.849418	12	8	20
Kočevska River, SLO	45.573	14.797	3	12	15
Bloščica River, SLO	45.786	14.516	5	19	24
Lake Prespa, ALB	40.864527	20.943473	16	38	54
Total			129	179	308

Results

This research included ten populations of *A. astacus* with a total of 308 specimens (179 males, 129 females). Populations with less than seven individuals were excluded from the analysis.

The *t* test showed that males and females were significantly different in measured morphometric characteristics (TL, ROL, ABL, TEL, CLL, CFL, CPL, CLH, CLW, CPW, ABW, TEW and in weight). Consequently, further analyses were carried out separately for both sexes.

One-way ANOVA results showed a significant difference in the majority of the measured morphometric characteristics among the male populations (F value $= 12.16$, $p < 0.001$) and also among female populations (F value $= 7.06$, $p < 0.001$). Males did not significantly differ among the populations only in the two morphometric characteristics (CLH and ABH), while females were no significantly different among the populations in four characteristics (ABL, CFL, CPH, ABW) ($p > 0.05$). Furthermore, results of Post hoc Bonferroni test indicated that significant differences exist in HEW between males from the Gazivode Reservoir and the Resnički Stream, Petrovačka River, Grliško Reservoir, Milošev fishpond, Lake Prespa ($p < 0.001$) and between the Gazivode Reservoir and the Kačer River ($p = 0.005$); in ROW between males from the Bloščica River and the Resnički Stream, Petrovačka River, Milošev fishpond, Lake Prespa, Kočevska River ($p < 0.001$); in CPW between males from the Kočevska River and all the other populations ($p < 0.001$); in ABW between the Gazivode Reservoir and the Resnički Stream ($p = 0.006$), between the Gazivode Reservoir and the Petrovačka River ($p = 0.001$), between the Gazivode Reservoir and the Kačer River ($p = 0.015$) and between males from the Gazivode Reservoir and the Milošev fishpond, Lake Prespa, Kočevska River, Bloščica River ($p < 0.001$).

Results of Post hoc Bonferroni test showed that significant differences for females exist in TL between populations from the Gazivode Reservoir and the Resnički Stream, Petrovačka River, Milošev fishpond ($p < 0.001$) and the Gazivode Reservoire and Lake Prespa ($p = 0.002$); in ROL between the Korenica Reservoir and the Resnički Stream, Milošev fishpond ($p < 0.001$) and the Korenica Reservoir and the Petrovačka River ($p = 0.001$); in TEL between populations from the Gazivode Reservoir and the Resnički Stream, Korenica Reservoir, Milošev fishpond ($p < 0.001$) and between the Gazivode Reservoir and the Petrovačka River ($p = 0.011$); in CPL between populations from Lake Prespa and the Resnički Stream ($p < 0.001$); in ABH between populations from the Korenica Reservoir and the Resnički Stream ($p = 0.001$), the Korenica Reservoir and the Petrovačka River ($p = 0.045$), the Korenica Reservoir and the Gazivode Reservoire ($p = 0.021$), and the Korenica Reservoir and the Milošev fishpond, Lake Prespa ($p < 0.001$); in HEW between populations from the Gazivode Reservoire and the Resnički Stream, Petrovačka River, Milošev fishpond, Lake Prespa ($p < 0.001$); in ROW between populations from the Korenica Reservoir and the Resnički Stream, Petrovačka River, Milošev fishpond, Lake Prespa ($p < 0.001$).

Since significant differences in measured morphometric characteristics between populations, for both males and females, were obtained, we conducted MDA. Multivariate discriminant analysis was performed in order to distinguish which of the measured morphometric characteristics contributes most to the difference among populations. The variables used in the analysis are listed in Table 2.

For male populations the first eight canonical discriminant functions were used in the analysis. The first two discriminant functions account for 52 and 18.4% of the explained variance, respectively, and the canonical R for those functions was 0.960 and 0.898, respectively. The

Table 2 Standardized canonical discriminant function coefficients for *A. astacus* males and females morphometric characteristics

Males				Females			
Morph. parameter	Function 1	Function 2	Function 3	Morph. parameter	Function 1	Function 2	Function 3
TL	0.260	0.577	0.170	TL	0.738	0.550	−0.062
ROL	−0.212	0.364	−0.372	ROL	0.132	0.169	−0.286
HEL	0.368	0.124	0.219	HEL	0.005	−0.197	−0.137
ABL	−0.433	−0.939	0.130	ABL	−1.216	−0.887	−0.516
TEL	−0.040	0.249	−0.126	TEL	−0.356	0.386	−0.104
CLL	−0.518	0.353	−1.613	CLL	0.153	0.902	−0.560
CFL	0.371	−0.247	0.243	CFL	0.124	−0.529	0.184
CPL	−0.077	0.504	−0.189	CPL	0.007	0.511	−0.239
CLH	0.021	−0.148	−0.024	CLH	0.141	−0.015	−0.119
CLW	−0.201	−0.520	0.428	CLW	−0.441	−0.845	0.245
CPH	0.205	−0.189	−0.376	CPH	0.207	−0.081	−0.338
ABH	0.185	0.097	−0.162	ABH	0.355	−0.223	−0.268
HEW	0.689	0.491	−0.433	HEW	0.440	0.412	0.753
ROW	0.312	0.271	0.462	ROW	0.835	−0.206	0.047
CGW	−0.092	−0.215	−0.212	CGW	−0.147	−0.582	−0.437
CPW	−0.507	0.546	0.523	CPW	−0.216	0.398	0.062
ARW	−0.036	0.151	0.314	ARW	0.054	−0.095	0.283
CEW	0.590	−0.358	0.004	CEW	−0.480	0.308	0.385
ABW	−0.269	−0.302	0.630	ABW	0.036	0.197	−0.313
TEW	−0.233	−0.254	−0.076	TEW	−0.164	−0.085	0.347
Weight	0.959	−0.179	1.131	Weight	−0.192	0.301	1.081
Eigenvalue	11.758	4.159	3.789	Eigenvalue	3.543	3.056	2.016
% of variance	52	18.4	16.8	% of variance	36.1	31.2	20.6
Cumulative %	52	70.4	87.2	Cumulative %	36.1	67.3	87.8
Canonical R	0.96	0.898	0.889	Canonical R	0.883	0.868	0.818

Eigenvalue, percentage of explained variance, cumulative percentage of the variance and canonical correlations are given as well

most important parameter for differentiation was weight (loading in discriminant function 1 = 0.959), followed by HEW (loading in discriminant function 1 = 0.689), CEW (loading in discriminant function 1 = 0.590) and CLL (loading in discriminant function 1 = −0.518). The second discriminant function account is weighed mostly by ABL (loading in discriminant function 2 = −0.939), TL (loading in discriminant function 2 = 0.577), CPW (loading in discriminant function 2 = 0.546) and CLW (loading in discriminant function 2 = 0.546).

For female populations, the first five canonical discriminant functions were used in the analysis. Canonical R for the first two discriminant functions had high values, and they were 0.883 and 0.868, respectively. The first discriminant function accounts for 36.1% of the explained variance and is weighed mostly by ABL (loading in discriminant function 1 = −1.216), ROW (loading in discriminant function 1 = 0.835) and TL (loading in discriminant function 1 = 0.738). The second discriminant function accounts for 31.2% of the explained variance and is weighed mostly by CLL (loading in discriminant function 2 = 0.902), ABL (loading in discriminant function 2 = −0.887) and CLW (loading in discriminant function 2 = −0.845).

We made plots of the first two discriminant functions for better visualization of separated populations for males (Fig. 2) and females (Fig. 3). For males, the first discriminant function provides discrimination between populations from Slovenia and other populations which have negative values for the first canonical function (Fig. 2). Since the first discriminant function is marked by high positive loadings for weight, HEW and CEW and high negative loadings for CLL, CPW and ABL (Table 2), we may say the higher the values of weight, HEW and CEW are, more likely the crayfish belongs to Slovenian populations, and the smaller the values of CLL, CPW and ABL are, more likely the crayfish belongs to Lake Prespa population and to Serbian populations. The second discriminant function

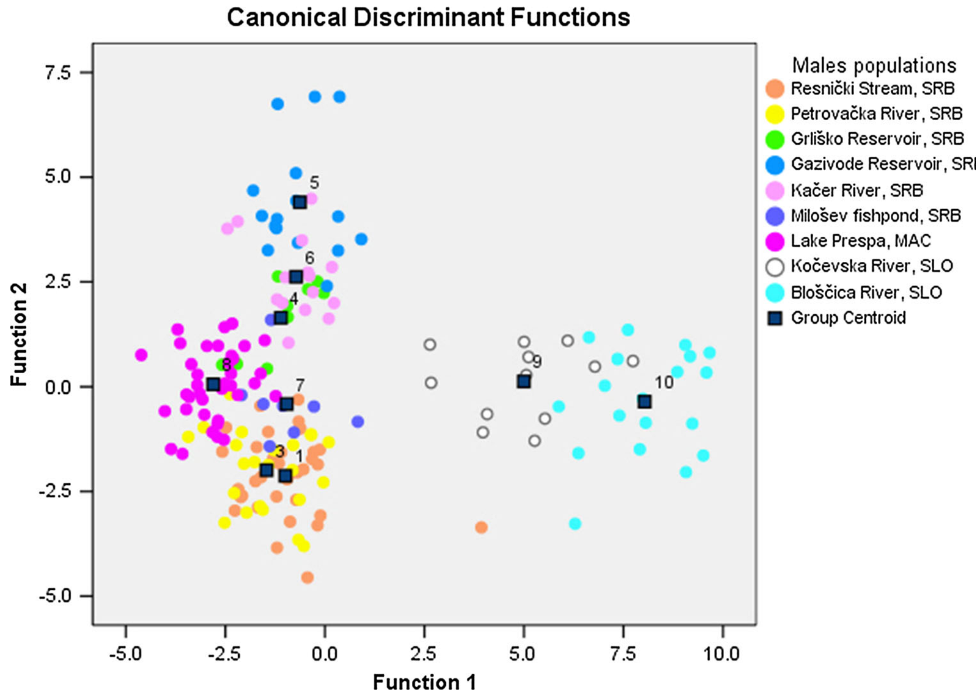

Fig. 2 Discrimination of *A. astacus* populations by the first two discriminant functions for males

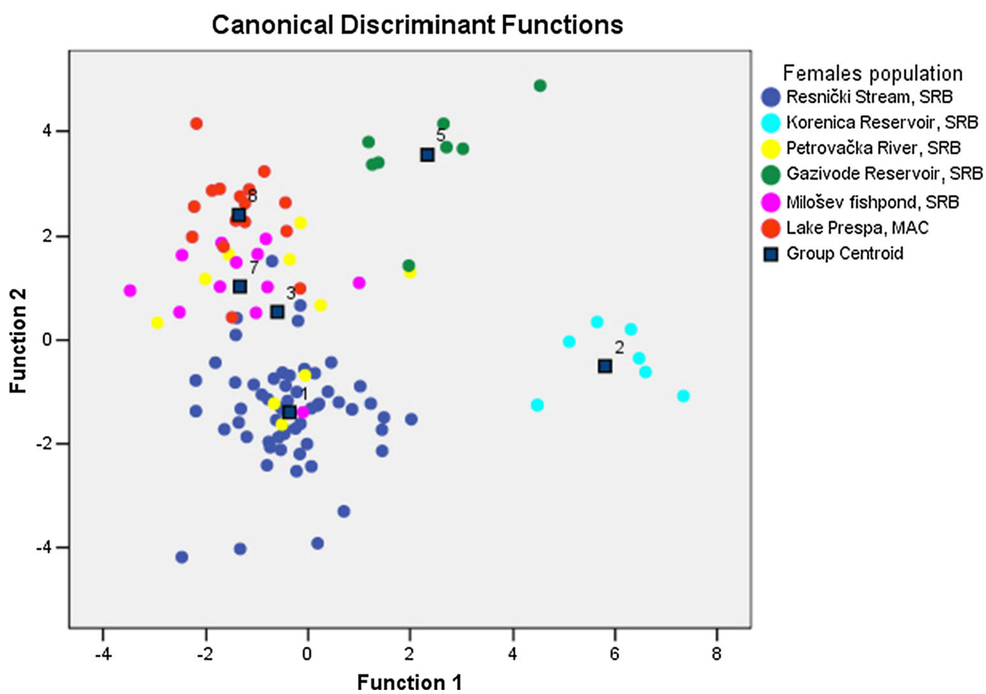

Fig. 3 Discrimination of *A. astacus* populations by the first two discriminant functions for females

seems to provide some discrimination between the Gazivode Reservoir population, Lake Prespa population and Petrovačka River population, but there was overlapping to some degree between the males from the Gazivode Reservoir and Kačer River, and males from the Petrovačka River, Resnički Stream and Miloševfishpond.

For females, the first discriminant function provides discrimination between populations from the Gazivode Reservoir and Korenica Reservoir and other populations which have negative values for the first canonical function (Fig. 3). The second discriminant function provides discrimination between populations from the Gazivode Reservoir and Resnički Stream. The first discriminant function is marked by high positive loadings for TL, ROW and HEW and high negative loadings for ABL, CEW and CLW (Table 2), so we may say the higher the values of TL, ROW and HEW are, more likely the crayfish belongs to the Korenica Reservoir and Gazivode Reservoir, and the smaller the values of ABL, CEW and CLW are, more likely the crayfish belongs to Lake Prespa population and to Milošev fishpond. The second discriminant function is marked by high positive loadings for CLL, CPL and TL and high negative loadings for ABL, CLW and CGW. The higher the values of CLL, CPL and TL are, more likely the crayfish belongs to population from the Gazivode Reservoir, and the smaller the values of ABL, CLW and CGW are, more likely the crayfish belongs to populations from the Resnički Stream.

The number of the correctly classified cases for males was 94.8 and 94.6% for females.

Discussion

Morphometrics are useful in order to quantify the size and shape of organisms with the methods of multivariate statistics (Klingenberg 2002). Traditionally, morphometrics was the application of multivariate statistical analyses to sets of quantitative variables such as length, width and height (Adams et al. 2004). Multivariate statistical analysis of morphometric characters is a powerful technique to investigate the geographical variation of stocks (Palma and Andrade 2002). Generally, morphological differentiation can appear as a consequence of genetic differences or environmental factors or their interaction (Pakkasmaa and Piironen 2001). The quantification of specific characteristics of an individual, or a group of individuals, can demonstrate the degree of speciation induced by both biotic and abiotic conditions and contribute to the definition of different stocks of species (Palma and Andrade 2002).

Research by Saila and Flowers (1969), Chambers et al. (1979), Sint et al. (2005, 2006, 2007), Maguire and Dakić (2011) had demonstrated that the analyses of a large morphometric data set per individual are a good tool for distinguish groups of decapods, which was not possible to achieve by analyses of only a few morphometric parameters (Grandjean and Souty-Grosset 2000). Morphometric characterization has also been applied successfully in freshwater and marine fish stock identification (Pakkasmaa and Piironen 2001; Palma and Andrade 2002).

In the present study, for the first time, we applied detailed morphometrics on distinct population of the noble crayfish from different regions of the Balkan Peninsula in order to verify whether there are differences between them. According to Albrecht (1983), the noble crayfish probably survived the last glaciations on the Balkan Peninsula and then spread northward and westward along the Danube drainage system (Schrimpf et al. 2011, 2014). The molecular study of Schrimpf et al. (2011, 2014) indicates high differentiation between populations from the western Balkans and the eastern Black Sea basin (Romania and Bulgaria) and identifies southeastern Europe as the hotspot of genetic diversity for the noble crayfish. Also, the authors suggest more detailed analysis of southeastern European populations to be conducted since many haplotypes that link the eastern Black Sea basin and western Balkans haplotypes are missing. Obtained results showed that differences exist between populations for both, males and females, in measured morphometric characteristic. Those differences reflect geographical distance, which is similar to findings by Sint et al. (2005) and Maguire and Dakić (2011), for other crayfish species. Similar to the abovementioned studies, we also found that males differed in more characters than females. The most important character for distinguishing males from different populations, with the highest loading in the discriminant analysis, was weight, which is different from previous studies of Maguire and Dakić (2011) and Sint et al. (2005). On the other hand, for females characteristics with the highest loading were ABL, CLL and CLW, likewise was in study of Maguire and Dakić (2011) for other crayfish species.

In our study, carapace measurements (HEW, ROW, CGW, CPW, CEW) had significantly contributed to discrimination between males and females populations, since their values had the highest loadings in the first and second discriminant functions. According to Sint et al. (2005), an advantage of carapace measurements is that they are neither influenced by loss and regeneration, such as the case with claws, nor by abdominal muscle contractions.

Recorded morphometric differences among Serbian populations were pronounced. Ascertainment differences may have been the result of the isolation of populations since Simić et al. (2008) had estimated that, in the period 1960–2006, the area inhabited by *A. astacus* on the territory of Serbia was reduced by more than 65% and only isolated populations have been registered. Population genetics theory predicts that small isolated populations with low levels of gene flow characteristically show a low genetic diversity within populations and a high genetic differentiation among populations (Grandjean and Souty-Grosset 2000). Besides the aforementioned degradation of habitats, pollution and disease that significantly contributed to population decline, Simić et al. (2008) also noted that *Astacus leptodactylus* behaves in an expansive way and substitutes *A. astacus* in some habitats. The results of Stucki and Romer (2001) also suggest that *A. leptodactylus* is more aggressive than *A. astacus* and is dominant in agonistic interactions.

In our study, the percentage of correctly classified specimens was higher than recorded in research by Sint et al. (2005), but similar to records in research by Maguire and Dakić (2011). The higher was the percentage of correct classification in the discriminant analysis, the greater was the distance between populations. Obtained result showed that the greatest distance appeared between the male populations from Slovenia and all other populations which is understandable considering the geographical distance between them and the loss of connectivity (Santos and Araújo 2015). When it comes to the female populations, the greatest distance exists between populations from Lake Prespa, Korenica Reservoir and Resnički Stream. This partly reflected the differences in the habitats. Morphometric differences obtained in this study could be a consequence of differences in noble crayfish genome or they may be attributed to their phenotypical plasticity. Part of the differences may be the consequence of aquatic ecosystem types and influences of local environmental factors—e.g., lakes and reservoirs versus rivers and streams (Santos and Araújo 2015). Environmental factors can produce phenotypic plasticity, which is the capacity of a genotype to produce different phenotypes in different environmental conditions (Pakkasmaa and Piironen 2001). It is well known that organisms tend to adapt to their specific environment, and these adaptations can influence both the genotype and the phenotype (Swain and Foote 1999). So to verify if recorded differences are of genetic origin, future studies should include molecular analyses (mitochondrial DNA and microsatellite analyses).

Once again, morphometric characteristics in combination with the discriminant analysis proved to be a good tool for distinguishing population. It is particularly important that this method is not invasive; it is inexpensive and can be easily applied in the field. Still, the knowledge of genetic variations within and between populations is needed as an essential prerequisite for the protection of species (Avise 2004; Skuza et al. 2016). Molecular phylogenetic research together with sound knowledge of morphometry will be a good basis for future conservation management of this endangered species.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animals rights statement All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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