Running head: Morphological diversity of the stone crayfish

Morphological evidence for hidden diversity in the threatened crayfish species *Austropotamobius torrentium*

Ivana Maguire[1]*, Nina Marn[2], Göran Klobučar[1]

[1]University of Zagreb, Faculty of Science, Department of Biology, Rooseveltov trg 6,
10000 Zagreb, Croatia

[2]Ruđer Bošković Institute, Bijenička cesta 54, 10000 Zagreb, Croatia

*Corresponding author, e-mail address: imaguire@zg.biol.pmf.hr

ABSTRACT

The stone crayfish *Austropotamobius torrentium* (Schrank, 1803) is the native European species with the distribution range in the Central and Southeast Europe. Recent molecular phylogenetic research has shown that within *A. torrentium* at least seven distinct monophyletic phylogroups exist, with the highest genetic diversity found within the northern-central Dinaric (NCD) region in Croatia. For some of these phylogroups, genetic divergence was sufficiently large to suggest that they may actually represent cryptic species. The focus of this research were the morphometric and meristic characteristics of stone crayfish populations of the Žumberak-Samoborsko gorje Nature Park (Croatia) situated in the genetically diverse NCD region. The aim was to test whether there are certain morphological features that clearly separate stone crayfish belonging to the three, previously identified, phylogroups into distinct groups based on morphology. For that purpose we analysed morphological data of stone crayfish belonging to three distinct populations inhabiting small streams within the Žumberak-Samoborsko gorje Nature Park. Analyses showed that significant differences in some of the recorded morphometric and meristic characteristics between studied populations, for both males and females, exist. Multivariate discriminant analyses of the measured morphological features revealed the characteristics that clearly separate populations in a similar way as molecular methods. For males these were the characteristics describing claws, carapace and rostrum, and for females those describing carapace, rostrum and total length. Percentage of correctly classified crayfish per population was high (91% - 100%) for both sexes. We discuss whether the morphological separations were congruent with the results previously obtained by molecular studies that have classified the three populations as three distinct phylogroups.

KEY WORDS stone crayfish; morphometric and meristic characteristics; multivariate statistics

INTRODUCTION

Crayfish importance in freshwater habitats food webs has been recognized for a long time (Gherardi et al., 2004; Nyström et al., 1996; Usio and Townsend, 2004), and they are regarded as a flagship species for comprehensive water protection (Füreder and Reynolds, 2003; Füreder et al., 2003).

The stone crayfish, *Austropotamobius torrentium* (Schrank, 1803), the smallest species of freshwater crayfish in the family Astacidae, is indigenous to the Central and South-eastern Europe in the waterbodies of the Black Sea drainage (Holdich et al., 2006; Kouba et al., 2014). It is a cold-adapted species that inhabits smaller lotic systems with rocky substrates on higher altitudes (Kouba et al., 2014). Within Croatia stone crayfish can be found in streams and rivers belonging to the Black Sea drainage, but some populations also exist within the Adriatic Sea drainage (Maguire and Gottstein Matočec, 2004; Maguire et al., 2011).

In the last few decades we are witnessing a pronounced trend of the stone crayfish populations' disappearance (Füreder et al., 2010; Maguire et al., 2011). This is largely due to a negative anthropogenic impact upon their natural habitats that are frequently isolated so, often, when a local population disappears, no natural re-colonisation can occur (Bohl, 1997; Chucholl and Schrimpf, 2016; Maguire et al., 2011). In addition to the anthropogenic and environmental stress, the stone crayfish are also endangered by the presence of invasive non-indigenous crayfish species that displace them from habitats due to high fertility, aggressiveness, flexible activity pattern, fast growth, and ability to transmit the disease crayfish plague that is lethal for indigenous crayfish (Chucholl and Schrimpf, 2016; Evans and Edgerton, 2002; Jussila et al., 2015). As a consequence, the stone crayfish is considered as a threatened species and is listed in Appendix III of the Bern Convention, in Annex II and V of Habitat directive (92/43/EEC). However, due to lack of sufficient data for the IUCN, the stone crayfish was designed to category “DD” or data deficient (Füreder et al., 2010). Still, in Croatia, due to historical and recent data on the stone crayfish distribution, number of populations and abundance (Maguire et al., 2011), it was possible to designate it to the National Red list of Crustacea in threat category as vulnerable (Gottstein et al., 2011).

In order to develop effective conservation and management plans, protection of existing crayfish populations and detection of suitable habitats in reserve areas for their reintroduction are a necessity (Peay, 2009; Streissl and Hödl, 2002). But prior to implementation of any conservation and management plans, research on the different important biological aspects of a chosen population, such as information on their breeding success (Maguire and Klobučar, 2011) or their genetics and morphology, should be undertaken. In these types of programmes it is essential to identify donor populations, which could be difficult when one comes upon highly similar lineages under a single nominal species (Bertocchi et al., 2008; Souty-Grosset and Reynolds, 2009; Taugbøl and Peay, 2004), as is the case of the stone crayfish that could be regarded, based on genetic diversity, as a species complex (Holdich et al., 2006); Klobučar et al., 2013; Trontelj et al., 2005).

The first large molecular phylogenetic research of the genus *Austropotamobius* indicated a distinct clade (phylogroup) within *A. torrentium* at the Upper Kupa (Kolpa) drainage in the northern Dinaric region (Trontelj et al., 2005). Recent comprehensive molecular phylogenetic and phylogeographic study using mitochondrial DNA of the stone crayfish sampled across the entire distribution range revealed existence of seven deeply divergent phylogroups within *A. torrentium*, with existence of five out of seven phylogroups, separated by pronounced genetic gaps, established in northern-central Dinaric (NCD) region. They were named according to their geographical landmarks: “Zeleni Vir” (ZV), “Gorski Kotar” (GK), “Žumberak, Plitvice and Bjelolasica” (ŽPB), “Lika and Dalmatia” (LD), “Banovina” (BAN) [all of which belong to the NCD region], “southern Balkan” (SB) and “central and south-eastern Europe” (CSE) (Klobučar et al., 2013) (Fig. 1). For at least four of these phylogroups (ZV, GK, ŽPB, and LD), genetic divergence (average values of uncorrected p-distances > 7%) was sufficiently large to suggest that they may actually represent cryptic species (Klobučar et al., 2013). Further, in the past, there were some morphological studies on the stone crayfish (Bott, 1950; Karaman, 1929; Karaman, 1961) intending to distinguish different populations, presumable *A. torrentium* subspecies, based on the specimens’ morphometric and meristic characteristics (Albrecht, 1982; Karaman, 1929; Karaman, 1961). The analyses were unreliable as they were made using a small number of characteristics and individuals, and no sufficiently stable diagnostic characters were proposed to distinguish different populations (Holdich et al., 2006). Apart from the previous research (Karaman, 1929; Karaman, 1961), no detailed studies on the morphology of the stone crayfish exist, and the only morphological diagnostic character that was suggested to distinguish stone crayfish belonging to the distinctive haplogroup detected in the northern Dinarides was a pronounced median rostral crista (Trontelj et al., 2005).

Knowing that genetic differences can have consequences in phenotypic appearance (Vogt et al., 2008), we hypothesised that observed genetic divergence could also be detected in morphological traits when a large set of morphological characteristics are analysed.

Contemporary research on a large number of morphometric characteristics per crayfish in combination with multivariate discriminant analysis has enabled researchers to discover significant differences between populations of the same species as well as between species (Bertocchi et al., 2008; Maguire and Dakić, 2011; Sint et al., 2005, 2006, 2007). Apart from the application of morphometric characteristics in distinguishing populations or crayfish species, some authors have also used crayfish meristic features, such as number of spines on the merus of the third maxilliped or presence of median rostral carina (Füreder and Machino, 2002; Harlioğlu, 2002; Karaman, 1961, 1962; Trontelj et al., 2005). Therefore, general purpose of this study was to supplement results of molecular analyses (Klobučar et al., 2013) with a detailed study of morphometric and meristic characteristics. To meet this goal, morphometric features of the stone crayfish belonging to three different populations/phylogroups within the Nature Park "Žumberak-Samoborsko gorje" were studied. The park is situated within the genetically diverse NCD region in the western Croatia, along the border with Slovenia, and it harbours four genetically distinct phylogroups (ZV, GK, ŽPB and CSE). At least three of these phylogroups (ZV, GK, and ŽPB) could represent cryptic species, based on the sufficiently large genetic divergence (Klobučar et al., 2013). The aims of this research were to determine 1) whether there are differences between the studied populations in recorded morphometric and meristic characteristics, and 2) whether possible differentiation of populations based on morphological features is congruent with the differentiation based on the molecular phylogentetic analyses performed previously on the individuals of the three populations belonging to ZV, GK, and ŽPB phylogroup. Results of this research could have a valuable contribution to the conservation and management programmes of the stone crayfish, not only in Croatia, but also in the whole area of distribution.

MATERIALS AND METHODS

The crayfish sampling was conducted in summer 2008, in three streams (Sopotski slap, Blate and Zeleni vir) within the Nature park Žumberak-Samoborsko gorje (surface area 333 km^2) that is situated in the western part of Croatia, along the Slovenian border (Fig. 1). All three streams are isolated, without current overground connection to a bigger river system, and direct geographic distances between them are: Sopotski slap – Blate 4.24 km; Sopotski slap – Zeleni vir 7.43 km; Blate – Zeleni vir 3.23 km (Vujnović, 2010). Before the field work, all of the required permits (working in the protected area, studying strictly protected species) were obtained from the legal authorities (Ministry of Environmental and Nature Protection, Public Institution for Management and Protection of Nature park Žumberak-Samoborsko gorje). The stone crayfish from those streams were chosen to be analysed for morphometric characteristics because sufficient number of individuals of both sexes were sampled there. Also, those populations were chosen because each of them represents a different phylogroup, or possibly even a separate cryptic species (Sopotski slap – ŽPB, Blate – GK, Zeleni vir – ZV), as shown by the results of phylogenetic study (Klobučar et al., 2013).

Fig. 1

The physico-chemical characteristics of water and habitat features of the studied streams were recorded on the sampling occasions (summer 2008) and were similar (Table 1).

Table 1

Crayfish were caught by hand or trapped with baited hand-made traps that have been placed along both banks of the stream and left there overnight.

Animals smaller than 5 cm total length were considered juvenile (Maguire and Klobučar, 2011; Streissl and Hödl, 2002), and therefore excluded from analyses to avoid introducing an additional source of variability by comparing juveniles and adults. Also, only uninjured and intermolt crayfish, in total 123 individuals, were examined. Number of females and males per population are shown in the table 2.

Table 2

For each crayfish 22 morphometric characteristics were recorded. Twenty-one characteristics were adopted from Sint et al. (2005): claw length (CLL), claw width (CLW), claw height (CLH), length of the claw palm (CPL), length of the claw finger (CFL), 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) and total length (TL). Extra measurement included cephalothorax length (CEF) (from postorbital to post lateral edge). All the characteristics were measured with a digital calliper with a 0.01 mm precision. Bilateral characteristics (CEF, CLL, CLW, CLH, CPL and CFL) were measured on both sides.

All of the measured morphometric characteristics were normalized for size by dividing them with the corresponding postorbital length (POL = HEL + ARL) (Sint et al., 2005), as a comparison of different sized animals could lead to misleading results (Chambers et al., 1979; Palma and Andrade, 2002).

An additional measure, describing lateral curvature of the carapace (angle α), was also included into the analyses (Sint et al., 2005).

Males and females were analysed separately because crayfish exhibit sexual dimorphism after attaining sexual maturity (Grandjean et al., 1997; Streissl and Hödl, 2002; Vlach and Valdmanová, 2015).

Meristic characteristics were examined under a magnifying glass, and for each crayfish three recorded characteristics included: number of spines on the ventral side of the merus of the third maxilliped, presence and pronunciation of rostral crista, and presence and type of denticulation (spines or tubercles) on the lower surface of the antennal exopod. Denticulation was recorded as number of spines or tubercles per crayfish, and then expressed as percentage of each denticulation type per population. Number of spines on the merus of the third maxilliped and denticulation on the antennal exopod are bilateral characteristics, and so were recorded for both body sides.

After examination, and tissue sampling for molecular study (Klobučar et al., 2013), crayfish were released back into the stream at the same position they were caught.

All the analyses were performed using statistical programmes Microsoft Excel 2007 and Statistica 8 for Windows (StatSoft.Inc.). T-test was applied to verify if there are significant differences in morphometric and meristic characteristics recorded for the left and the right body side, and also to compare if two sexes significantly differ in the recorded meristic characteristics. Pearson correlation coefficient was used to verify if there are significant correlations between morphometric and meristic characteristics. To verify if there are differences in recorded meristic characters (ordinal variables) between populations nonparametric Kruskal-Wallis ANOVA and chi-square test were used (cf. Zar, 1996). ANOVA with Bonferroni post-hoc test was applied to verify if there are significant differences between populations in measured morphometric characteristics. Multivariate discriminant analysis was applied to get differentiation of populations, based on measured morphometric characteristics. From the selected morphometric data sets the stepwise method was used to single out the characteristics that make the most significant contribution to the discrimination. Once a model was finalised with the derived discriminant, classification function was used to determine to which group each case (individual) most likely belongs. To visualise the results of the analyses scatterplots for the two discriminant functions were produced. Also, Mantel test was applied to establish correlations between geographic, morphometric and genetic distances (Mantel, 1967). For geographic distances we used direct geographic distances between the streams, due to reasons mentioned on the beginning of this chapter. For morphometric distances, we used the Euclidian distances calculated for measured morphometric features between the populations. Pairwise genetic distances between populations were calculated from the concatenated data set (Klobučar et al., 2013). Mantel tests were performed for three matrices: i) between morphometric distance and genetic distance, ii) between morphometric distance and stream distance, iii) between genetic distance and stream distance, and iv) between all three matrices. Mantel tests were performed in the MEGA6 (Tamura et al., 2013) using 9999 permutations.

RESULTS

Morphometrics

There are no differences in bilateral morphometric characteristics recorded for the left and the right side of the body (CLL – t = 1.59, p = 0.12; CFL – t = 1.14, p = 0.26; CPL – t = 1.27, p = 0.21; CLW – t = 1.23, p = 0.22; CLH – t = 1.13, p = 0.26; CEF – t = 0.17, p = 0.87). So in the further analyses right body side measurements were used.

Significant differences between populations in measured morphometric characteristics exist for both males (F = 5.43; p < 0.01) and females (F = 5.27; p < 0.01). Males significantly differ in ROL between Blate and Zeleni vir (p < 0.01), and Blate and Sopotski slap (p = 0.01), in CPH between Blate and Zeleni vir (p = 0.01), and Blate and Sopotski slap (p < 0.01), and in TEW between Blate and Sopotski slap (p = 0.03). Females differ significantly in CLW between Zeleni vir and Sopotski slap ($p = 0.02$), in ROL between Blate and Zeleni vir ($p < 0.01$), and Blate and Sopotski slap ($p < 0.01$), in ROW between Blate and Zeleni vir ($p = 0.02$), and in carapace curvature (angle α) between Blate and Zeleni vir ($p = 0.02$), and Zeleni vir and Sopotski slap ($p < 0.01$).

Results of multivariate discriminant analysis singled out that for males the most important discriminant characteristics, with the highest loadings in discriminant functions, were those describing claws (CLW, CLL, CFL, CPL), cephalothorax (CPH) and rostrum (ROL and ROW) (Table 3). For females the highest loadings in discriminant functions were obtained for the characteristics describing carapace (CPW, CEW, α, CPH, CEF, ARW), rostrum (ROL) and total length (TL) (Table 4).

Table 3

Table 4

Scatterplots for the two discriminant functions are shown in Fig. 2 (males) and Fig. 3 (females). The first discriminant function discriminates well males from the Sopotski slap and the Zeleni vir populations, while the second discriminant function discriminates Blate males from males of Sopotski slap and Zeleni vir populations. As the first discriminant function is marked by high negative loadings for CLW and CLL (Table 2), we may say that the smaller the values of CLW and CLL, the more likely it is that the males belong to the population from Sopotski slap. Also, as the same function is marked by high positive loadings for CFL, CPL and ROL (Table 2), for the higher values of CFL, CPL and ROL, it is more likely that the males belong to the populations from Zeleni vir. In the same way the discrimination for the second discriminant function between the Blate males and the rest of populations (Sopotski slap and Zeleni vir) can be explained; the higher the values of ROW, CFL, CPH and CLW are, the more likely it is that the males belong to populations from Sopotski slap or Zeleni vir, and the lower the value of CLL, the bigger is the chance that males belong to the population from Blate.

Fig. 2

For females, the first discriminate function, same as in males, discriminates crayfish belonging to the Sopotski slap population from those belonging to the Zeleni vir population. The second discriminant function discriminates well females from the Blate population from the rest of populations.

Fig. 3

As the first discriminant function is marked by high negative loadings for CPW, carapace curvature (α) and ARW (Table 3), we may say that the smaller the values are of those characteristics, the more likely it is that females belong to the population from Zeleni vir, and the higher the values are of TL and CPH (high positive loadings in the first discriminant function), the more likely it is that the females belong to the population from Sopotski slap. The second discriminant function is marked by high positive loadings for CPW, ABW and CPH, so we may say that the higher values for those characters are, the more likely it is that the female belongs to the population from Sopotski slap or Zeleni vir, and the smaller the values of TL, CEF and CEW are (high negative loadings in the second discriminant function), then the probability is higher that the female belongs to the population from Blate.

The number of correctly classified cases for both males and females was high (Table 5).

Table 5

Mantel tests showed positive correlations between geographic and genetic distance (Mantel's R = 0.51) and positive partial correlation among all three distance matrices (Mantel's partial R = 1). Negative correlations were obtained for genetic-morphometric distances (Mantel's R

= -0.16) and morphometric-geographic distances (Mantel's R = -0.93). Still, none of the correlations were significant.

Meristics

There is no significant difference between males and females neither in the number of spines on the third maxilliped ($t = 0.675$, $p = 0.501$), nor in the number of spines and tubercles on the lower surface of antennal exopod ($t = -1.99$, $p = 0.054$, and $t = 0.939$, $p = 0.375$ for spines and tubercles respectively). Therefore males and females were pooled for further analyses. There was neither a significant difference in the number of spines on the left and the right merus of the third maxilliped ($t = 1.384$, $p = 0.172$), nor in the number of spines or tubercles on the lower surface of the left and the right antennal exopod ($t = -0.441$, $p = 0.662$, and $t = -1.846$, $p = 0.203$ for spines and tubercles respectively). Therefore only the right body side data were analysed. Also, no significant correlation was found either between crayfish total length and the number of spines on the third maxilliped ($r = 0.18$, $p > 0.01$), or between TL and number of spines/tubercles on the lower surface of the antennal exopod ($r = 0.07$, $p > 0.05$, $r = 0.08$, $p > 0.05$ for spines and tubercles respectively).

A significant difference in the number of spines on the third maxilliped between populations exists ($H_{(2, 121)} = 38.21$, $p < 0.001$) (Fig. 4). Significant differences were recorded between populations from Blate and Sopotski slap ($z = 4.045$, $p < 0.001$), and Blate and Zeleni vir ($z = 5.66$, $p < 0.001$).

Fig. 4

All of the crayfish examined had denticulation on the lower surface of antennal exopod, and variation in shape of denticles (spines or tubercles) was recorded (number of animals with spines, number of animals with tubercles) within each population (Fig. 5), but no statistically significant difference between populations was found ($\chi^2 = 2.02$, $p = 0.36$).

Fig. 5

All of the crayfish examined had a rostral carina present; however, within the populations there was observed variation in the development of the carina, with the majority of crayfish from the Blate population having the strong carina (Fig. 6). Statistically significant difference in the carina's strength was observed ($\chi^2 = 9.43$, $p < 0.01$).

Fig. 6

DISCUSSION

Research on the large sets of morphometric as well as meristic characteristics, in combination with multivariate statistics have proven to be a successful tool in the analyses of possible differences between and within species (Bertocchi et al., 2008; Costa et al., 2003; Grandjean and Souty-Grosset, 2000; Maguire and Dakić, 2011; Sint et al., 2005, 2006, 2007). They have the advantage of being relatively fast and easily applicable in the field, not harmful for studied specimens and complementary to the genetic (Bertocchi et al., 2008; Fevolden and Hessen, 1989; Sint et al., 2007) and ecological research (Inoue et al., 2013).

Sint et al. (2007) found a positive relationship between geographical distance and the morphological divergence between studied stone crayfish populations from the Tyrol, Austria. However, our study reveals relatively high morphological variation among three geographically close, and genetically distinct, *A. torrentium* populations. For males, discrimination between populations was based on the characteristics describing claws, cephalothorax and rostrum, whereas for females discrimination between populations was based on the shape of cephalothorax, and females' body length. Similar characteristics (claws and cephalothorax) were found to be discriminant between *A. torrentium* populations from North Tyrol (Austria) in the research by Sint et al. (2007), whereas the rostrum was a discriminative characteristic that separates well populations of the white-clawed crayfish in Italy (Bertocchi et al., 2008).

Two out of three meristic characteristics were useful in the separation (distinction) of populations: the number of spines on the third maxilliped and the strength of median rostral carina. The recorded number of spines on the third maxilliped is in accordance with the numbers found by Karaman (1961, 1962). Our results showed that significant difference in the number of spines between populations exist, with the population from Zeleni vir possessing the highest number of spines, and the population from Blate having the smallest number (Fig. 4). Therefore this characteristic seems to be reliable as a character for separating different populations.

In the research by Trontelj et al. (2005) it has been found that *A. torrentium* specimens from the Upper Kupa basin (border area between Croatia and Slovenia, positioned to the south from the Žumberak-Samoborsko gorje NP) have a very strong and pronounced median rostral carina. Results of our research (Fig. 6) showed that variability in the pronunciation of carina exists within populations. In the Blate population the majority of specimens had strongly pronounced carina, which is similar to the findings of Trontelj et al. (2005). According to the molecular phylogenetic results (Klobučar et al., 2013), populations from the Kupa River basin (Trontelj et al., 2005) and Blate are clustered together into the phylogroup Gorski kotar (GK). Thus, the presence of pronounced median rostral carina in the majority of studied specimens belonging to those populations, single out the strength of the rostral carina as a meristic characteristic that is probably typical of analysed stone crayfish from GK phylogroup, and a trait that can be used in distinguishing them from crayfish belonging to other genetically different groups.

In general, crustaceans are known to exhibit high morphological plasticity (Wills, 1998). Phenotypic variation in morphometric and meristic characteristics between different populations of the same crayfish species were previously recorded (Buhay et al., 2007; Grandjean and Souty-Grosset, 2000; Haddaway et al, 2012; Rudolph et al., 2016). Observed variations could be a consequence of either environmental (Austin and Knott, 1996; Ghia et al., 2006; Grandjean and Souty-Grosset, 2000; Haddaway et al, 2012; Rudolph et al., 2016; Sint et al., 2005, 2006) or genetic factors (Buhay et al., 2007; Cataudella et al., 2010; Maguire et al., 2014; Sint et al., 2007), but probably both environmental and genetic mechanisms play a role in the final phenotypic outcome as it was found in the research on fish (Begg et al., 1999; Imre et al., 2002; Jerry and Cairns, 1998; Pakkasmaa and Piironen, 2001; Swain and Foot, 1999) and crayfish (Baric et al., 2005a, b; Bertocchi et al., 2008; Fevolden and Hessen, 1989; Mathews et al., 2008).

In our research all three populations live in separate, isolated streams within a relatively small geographical area. We could not statistically compare the recorded environmental conditions (altitude, substrate of the bottom, water velocity, and surrounding environment) of their streams/locations due to insufficient data size (a single measurement). However, an overview of their habitats' characteristics (Table 1) suggests that the conditions in the streams are similar. The differences found between morphometric and meristic characteristics therefore could not be solely attributed to the adaptation to local environmental conditions. The similarity of environmental conditions, in the context of the previously identified distinct phylogroups (Klobučar et al., 2013), thus may suggest that the recorded differences in morphometric and meristic characteristics between populations could be genetically based. But, since no significant correlations were observed between morphometric and genetic distances, this assumption could not be confirmed.

A similar relationship between the results of morphometric and genetic research was found in the studies on other terrestrial (Brehm et al., 2001) and freshwater species (e Silva et al., 2008; Inoue et al., 2013), including the white-clawed crayfish populations from France (Grandjean and Souty-Grosset, 2000), or from Italy (Ghia et al., 2006). Although there are studies that found a significant relationship between morphological and genetic distances of white-clawed from Italy and Austria (Baric et al., 2005a, b; Bertocchi et al., 2008; Scalici and Bravi, 2011). Absence of correlations between morphometric and genetic features point to the fact that morphological appearance is not primary controlled by genetic, and that phylogenetic based on mtDNA presents just a segment of taxon's evolutionary past (Inoue et al., 2013).

Morphological differences between studied populations were established by applying discriminant analyses on a large morphometric data set and they are not obvious on the first sight and cannot be used on a single specimen. Possible explanation for less pronounced (more subtle) morphological differences between different stone crayfish populations chosen for this study could be attributed to the fact that stone crayfish are cold-adapted species, and organisms adapted to harsh, stable conditions (such is constant cold water) can reduce or eliminate morphological changes that would normally accompany speciation (Bickford et al., 2006).

The results of this research confirm the potential to implement morphometric and meristic studies of large data sets for identification and distinction of stone crayfish populations, or other possibly cryptic species, in future protection and management projects.

We have concluded that the water bodies within a relatively small geographical area of the Nature Park "Žumberak Samoborsko gorje" are inhabited by three morphometrically, and meristically distinct stone crayfish groups, and should be given high priority in both shortand long-term conservation measures. Still, since no significant correlations were found among distance matrices, analyses of additional samples from a wider geographic range, as well as additional genetic markers (e.g. microsatellite loci) are needed to get a clear picture on the relations among divergent stone crayfish populations.

ACKNOWLEDGEMENTS

This work was funded by the Nature Park Žumberak Samoborsko gorje. The authors are grateful to Prof. D. M. Holdich for valuable comments and English language corrections, and to Dr. M. Jelić for statistical analyses assistance.

REFFERENCES

Albrecht, H. 1982. Das System der europäischen Flußekrebse (Decapoda, Astacidae): Vorschlag und Begründung. Mitteilungen aus den Hamburgischen Zoologischen Museum und Institut 79: 187-210.

Austin, C. M., and B. Knott. 1996. Systematics of the Freshwater Crayfish Genus *Cherax* Erichson (Decapoda: Parastacidae) in South-Western Australia: Electrophoretic, Morphological and Habitat Variation. The Australian Journal of Zoology 44: 223- 258.

Baric, S., A. Höllrigl, L. Füreder, and J. Dalla Via. 2005a. Mitochondrial and microsatellite DNA analyses of *Austropotamobius pallipes* populations in South Tyrol (Italy) and Tyrol (Austria). Bulletin Français de la Pêche et de la Pisciculture 376-377: 599-612.

--------, A. Höllrigl, C. Kerschbamer, L. Füreder, J. Petutschnig, and J. Dalla Via. 2005b. Update of the molecular phylogeny of the *Austropotamobius pallipes* species complex by including specimens from South Tyrol (Italy) and Carinthia (Austria). Bulletin Français de la Pêche et de la Pisciculture 376-377: 627-636.

Begg, G. A., K. D. Friedland, and J. P. Pearce. 1999. Stock identification and its role in stock assessment and fisheries management: and overview. Fisheries Research 43: 1-8.

Bertocchi, S., S. Brusconi, F. Gherardi, A. Buccianti, and M. Scalici. 2008. Morphometrical characterization of the *Austropotamobius pallipes* species complex. The Journal of Natural History 42: 2063-2077.

Bickford, D., D. J. Lohman, N. S. Sodhi, P. K. L. Ng, R. Meier, K. Winker, K. K. Ingram, and I. Das. 2006. Cryptic species as a window on diversity and conservation. Trends in Ecology & Evolution 22(3): 148-155.

Bohl, E. 1997. An isolated population of the white-clawed crayfish *(Austropotamobius pallipes*) in the principality of Liechtenstein. Bulletin Français de la Pêche et de la Pisciculture 347: 701-712.

Bott, R. 1950. Die Flusskrebse Europas (Decapoda, Astacidae). In, R. Martens (ed), Abhandlungen der Senckenbergischen Naturforschenden Gesellschaft, Vol. 483. Kremer, Frankfurt a. M.: 4–36.

Brehm, A., M. Khadem, J. Jesus, P. Andrade, and L. Vicente. 2001. Lack of congruence between morphometric evolution and genetic differentiation suggests a recent dispersal and local habitat adaptation of the Madeiran lizard *Lacerta dugesii.* Genetics Selection Evolution 33: 671-685.

Buhay, J. E., G. Moni, N. Mann, and K. A. Crandall. 2007. Molecular taxonomy in the dark: Evolutionary history, phylogeography, and diversity of cave crayfish in the subgenus *Aviticambarus*, genus *Cambarus*. Molecular Phylogenetics and Evolution 42: 435-448.

Cataudella, R., M. Paolucci, C. Delaunay, A. H. Ropiquet, M. Baslamo, and F. Grandjean. 2010. Genetic variability of *Austropotamobius italicus* in the Marches region: implications for conservation. Aquatic Conservation: Marine and Freshwater Ecosystems 20: 261-268.

Chambers, C. L., J. F. Payne, and M. L. Kennedy. 1979. Geographical variation in the dwarf crayfish, *Cambarellus puer* Hobbs (Decapoda, Cambaridae). Crustaceana 36: 39-55.

Chucholl, C., and A. Schrimpf. 2016. The decline of endangered stone crayfish (*Austropotamobius torrentium*) in southern Germany is related to the spread of invasive alien species and land-use change. Aquatic Conservation: Marine and Freshwater Ecosystems 26(1): 44-56.

Costa J. L., P. R. De Almeida, and M. J. Costa. 2003. A morphometric and meristic investigation of Lusitanian toadfish *Halobatrachus didactylus* (Bloch and Schneider, 1801): evidence of population fragmentation on Portuguese coast. Scientia Marina 67(2): 19-231.

E Silva, D. de M., A. D. da Cruz, R. P. Bastos, M. P. de C. Telles, and J. A. F. Diniz-Filho. 2008. Morphometric and genetic differentiation among populations of *Eupemphix nattereri* (Amphibia, Anura, Leiuperidae) from central Brazil. Iheringia, Série Zoologia 98(4): 493-500. http://dx.doi.org/10.1590/S0073-47212008000400013

Evans, L. H., and B.F. Edgerton. 2002. Pathogens, parasites and commensals. In, D. M. Holdich (ed), Biology of freshwater crayfish. Blackwell science, Oxford: 377-438.

Fevolden, S. E., and D. O. Hessen. 1989. Morphological and genetic differences among recently founded populations of noble crayfish (*Astacus astacus*). Hereditas 110: 149-158.

Füreder, L., and Y. Machino. 2002. A revised determination key of freshwater crayfish in Europe. Berichte des naturwissenschaftlich-medizinischen Vereins in Innsbruck 89: 169-178.

--------, B. Oberkofler, R. Hanel, J. Leiter, and B. Thaler. 2003. The freshwater crayfish *Austropotamobius pallipes* in South Tyrol: Heritage species and bioindicator. Bulletin Français de la Pêche et de la Pisciculture 370-371: 79-95.

-------- and J. D. Reynolds. 2003. Is *Austropotamobius pallipes* a good bioindicator? Bulletin Français de la Pêche et de la Pisciculture 370-371: 157-163.

--------, F. Gherardi, and C. Souty-Grosset. 2010. *Austropotamobius torrentium*. In IUCN 2010. IUCN Red List of Threatened Species. Version 2015-4. <www.iucnredlist.org>. Downloaded on 03 March 2016.

Gherardi, F., P. Acquistapace, and G. Santini. 2004. Food selection in omnivores: a case study of the crayfish *Austropotamobius pallipes*. Archiv fur Hydrobiologie 159: 357-376.

Ghia, D., P.A. Nardi, A. Negri, A. Bernini, A. Bonardi, G. Fea, and M. Spairani. 2006. Syntopy of *A. pallipes* and *A. italicus*: genetic and morphometrical investigation. Bulletin Français de la Pêche et de la Pisciculture 380-381: 1001-1018.

Gottstein S., S. Hudina, A. Lucić, I. Maguire, I. Ternjej, and K. Žganec. 2011. Crveni popis rakova (Crustacea) slatkih i bočatih voda Hrvatske. Državni zavod za zaštitu prirode, Zagreb: 51.

Grandjean, F., D. Romain, C. Avila-Zarza, M. Bramard, C. Souty-Grosset, and J.P. Mocquard. 1997. Morphometry, sexual dimorphism and size at maturity of the white-clawed crayfish *Austropotamobius pallipes pallipes* (Lereboullet) from a wild French population at Deux-Sëvres (Decapoda, Astacidae). Crustaceana 70: 31-44.

-------- and C. Souty-Grosset. 2000. Genetic and morphological variation in the endangered crayfish species *Austropotamobius pallipes* (Lereboullet) (Crustacea Astacidae) from the Poitou-Charentes region (France). Aquatic Sciences 62: 1-19.

Haddaway, N. R., R. J. G. Mortimer, M. Christmas, J. W. Grahame, and A. M. Dunn. 2012. Morphological diversity and phenotypic plasticity in the threatened British white-clawed crayfish (*Austropotamobius pallipes*). Aquatic Conservation: Marine and Freshwater Ecosystems 22: 220-231.

Harlioğlu, M. M. 2002. Differences in the crista dentata of the ishium of the third maxilliped in *Astacus leptodactlyus* (Eshscholtz, 1823). Folia Zoologica 51: 111-116.

Holdich, D.M., P. Haffner, and P. Y. Noël. 2006. Species files. In Souty-Grosset, C., D. M. Holdich, P. Y. Noël, J. D. Reynolds, and P. Haffner (eds), Atlas of Crayfish in Europe. Muséum national d'Histoire naturelle, Paris: 49-131.

Imre, I., R. L. McLoughlin, and D. G. L. Noakes. 2002. Phenotypic plasticity in brook charr: changes in caudal fin induced by water flow. Journal of Fish Biology 61: 1171-1181.

Inoue, K., D. M. Hayes, J. L. Harris, and A. D. Christian. 2013. Phylogenetic and morphometric analyses reveal ecophenotypic plasticity in freshwater mussels *Obovaria jacksoniana* and *Villosa arkansasensis* (Bivalvia: Unionidae). Ecology and Evolution 3(8): 2670-2683.

Jerry, D. R., and S. C. Cairns. 1998. Morphological variation in the catadromous Australian bass from seven geographically distinct riverine drainages. Journal of Fish Biology 52: 829-843.

Jussila, J., I. Maguire, H. Kokko, and J. Makkonen. 2015. Chaos and Adaptation in the Pathogen-Host Relationship in Relation to the Conservation - The Case of the Crayfish Plague and the Noble Crayfish. In Kawai T., Z. Faulkes, and G. Scholth (eds) Freshwater crayfish: a global overview. CRC Press, Boca Raton: 246-275.

Karaman, M. S. 1961. Slatkovodni rakovi Jugoslavije. Ribarstvo Jugoslavije 3: 1-33.

-------- 1962. Ein Beitrag zur Systematik Astacidae (Decapoda). Crustaceana 3: 173-191.

Karaman, S. 1929. Die Potamobiiden Jugoslviens. Glasnik zemaljskog muzeja u Bosni i Hercegovini XLI: 147-150.

Klobučar, G., M. Podnar, M. Jelić, D. Franjević, M. Faller, A. Štambuk, S. Gottstein, V. Simić, and I. Maguire. 2013. Role of the Dinaric Karst (western Balkans) in shaping the phylogeographic structure of the threatened crayfish *Austropotamobius torrentium*. Freshwater Biology 58: 1089-1105.

Kouba, A., A. Petrusek, and P. Kozák. 2014. Continental-wide distribution of crayfish species in Europe: update and maps. Knowledge and management of aquatic ecosystems 413: 05. doi: 10.1051/kmae/2014007.

Maguire, I., and S. Gottstein Matočec. 2004. The distribution pattern of freshwater crayfish in Croatia. Crustaceana 77: 25-49.

--------, M. Jelić, and G. Klobučar. 2011. Update on the distribution of freshwater crayfish in Croatia. Knowledge and management of aquatic ecosystems 401: 31. doi: 10.1051/kmae/2011051.

-------- and G. Klobučar. 2011. Size structure, maturity size, growth and condition indeks of stone crayfish (*Austropotamobius torrentium*) in North-West Croatia. Knowledge and management of aquatic ecosystems 401: 12. doi: 10.1051/kmae/2011026.

-------- and L. Dakić. 2011. Comparative analyses of *Astacus leptodactylus* morphological characteristics from Croatia and Armenia. Biologia, Section zoology 66(3): 491-498.

--------, M. Podnar, M. Jelić, A. Štambuk, A. Schrimpf, H. Schulz, G. Klobučar. 2014. Two distinct evolutionary lineages of the *Astacus leptodactylus* species-complex (Decapoda : Astacidae) inferred by phylogenetic analyses. Invertebrate Systematics 28(2): 117-123.

Mantel, N. 1967. The detection of disease clustering and a generalized regression approach. Cancer Research 27: 209–220.

Mathews, L.M., L. Adams, E. Anderson, M. Basile, E. Gottardi, and M.A. Buckholt. 2008. Genetic and morphological evidence for substantial hidden biodiversity in a freshwater crayfish species complex. Molecular Phylogenetics and Evolution 48: 126-135.

Nyström, P., C. Brönmark, and W. Granèli. 1996. Patterns in benthic food webs: a role for omnivorous crayfish? Freshwater Biology 36: 631-646.

Pakkasmaa, S., and J. Piironen. 2001. Morphological differentiation among local trout (*Salmo trutta*) populations. Biological Journal of the Linnean Society 72: 231-239.

Palma, J., and J. P. Andrade. 2002. Morphological study of *Diplodus sargo*, *Diplodus puntazzo* and *Lithognatus morryrus* (Sparidae) in the Eastern Atlantic and Mediterranean Sea. Fisheries Research 57: 1-8.

Peay, S. 2009. Invasive non-indigenous crayfish species in Europe: Recommendations on managing them. Knowledge and management of aquatic ecosystems 394-395: 03. doi: 10.1051/kmae/2010009.

Rudolph, E., N. Colihueque, and M. Yañez. 2016. Morphological and genetic analysis in morphologically divergent river and lake specimens of the freshwater crayfish *Samastacus spinifrons* (Philippi, 1882) (Decapoda, Parastacidae). Crustaceana 89(8): 877-899.

Scalici, M., and R. Bravi. 2011. Solving alpha-diversity by morphological markers contributes to arranging the systematic status of a crayfish species complex (Crustacea, Decapoda). Journal of Zoological Systematics and Evolutionary Research doi: 10.1111/j.1439-0469.2011.00643.x.

Schrank, F. 1803. Fauna Boica Durchgedachte geschichte der in Baiern einheimischen und zahmen Thiere. Steinschen buchandlung, Nürnberg 3: viii + 272 pp.

Sint, D., J. Dalla Via, and L. Füreder. 2005. Morphological variations in *Astacus astacus* L. and *Austropotamobius pallipes* (Lereboullet) populations. Bulletin Français de la Pêche et de la Pisciculture 376–377: 637–652.

--------, J. Dalla Via, and L. Füreder. 2006. The genus *Austropotamobius* in the Ausserfern region (Tyrol, Austria) with an overlap in the distribution of *A. torrentium* and *A. pallipes* populations. Bulletin Français de la Pêche et de la Pisciculture 380-381: 1029-1040.

--------, J. Dalla Via, and L. Füreder. 2007. Phenotypical characterization of indigenous freshwater crayfish populations. Journal of Zoology 273: 210–219.

Souty-Grosset, C., and J. D. Reynolds. 2009. Current ideas on methodological approaches in European crayfish conservation and restocking procedures. Knowledge and management of aquatic ecosystems 394-395: 01 doi: 10.1051/kmae/2009021.

Streissl, F., and W. Hödl. 2002. Growth, morphometrics, size at maturity, sexual dimorphism and condition index of *Austropotamobius torrentium* Schrank. Hydrobiologia 477: 201-208.

Swain, D. P., and C. J. Foote. 1999. Stock and chameleons: the use of phenotypic variation in stock identification. Fisheries Research 43: 113–128.

Tamura, K., G. Stecher, D. Peterson, A. Filipski, and S. Kumar. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0.. Molecular Biology and Evolution 30(12): 2725-9. doi: 10.1093/molbev/mst197.

Taugbøl, T., and S. Peay. 2004. Reintroduction of native crayfish and habitat restoration. Bulletin Français de la Pêche et de la Pisciculture 372-373: 465-471.

Trontelj, P., Y. Machino, and B. Sket. 2005. Phylogentic and phylogeographic relationships in the crayfish genus *Austropotamobius* inferred from mitochondrial COI gene sequences. Molecular Phylogenetics and Evolution 34: 212-226.

Usio, N., and C. R. Townsend. 2004. Roles of crayfish: consequences of predation and bioturbation for stream invertebrates. Ecology 85: 807-822.

Vlach, P., and L. Valdmanová. 2015. Morphometry of the stone crayfish (*Austropotamobius torrentium*) in the Czech Republic: allometry and sexual dimorphism. Knowledge and Management of Aquatic Ecosystems 416: 16 doi: 10.1051/kmae/2015012

Vogt, G., M. Huber, M. Thiemann, G. van den Boogaart, O. J. Schmitz, and C. D. Schubart. 2008. Production of different phenotypes from the same genotype in the same environment by developmental variation. Journal of Experimental Biology 211: 510-523.

Vujnović, T. 2010. Hidrološke značajke Parka prirode „Žumberak-Samoborsko gorje“. Doctoral thesis, University of Zagreb, 168 pp (in Croatian).

Wills, M. A. 1998. Arhtopod Relationships, pp. 189-211. In, Fortey, R. A., and R. H, Thomas (eds.), A phylogeny of recent and fossil Crustacea derived from morphological characters. Springer-Science+Business media.

Zar, J. H. 1999. Biostatistical Analysis . Prentice Hall, Upper Saddle River, NJ, 662 pp.

Table 1 Characteristics of studied streams: altitude (in meters above sea level), stream width (in meters), stream depth (in centimetres), percentage of shade over a stream, O_2 - oxygen concentration in mg/L, pH, T – water temperature in °C, composition of the substrate on the bottom of stream, expressed in percentage.

Location	Altitude	Width	Depth	Shade	O_2	pH	T	Substrate of the bottom		
								Stones	Pebbles	Send
Blate Stream	654	0.5-2	10-60	95	6.8	8.31	13.2	10	15	75
Zeleni vir Stream	606	1.5-3	10-50	95	8.64	8.2	14.5	50	30	20
Sopotski slap Stream	555	0.5-1	10-50	80	9.38	7.3	15.3	50	25	25

Table 2 Number of males and females examined per location/population.

Location / population	Males	Females
Blate Stream	27	31
Zeleni vir Stream	17	13
Sopotski slap Stream	17	18

Table 3 Results of discriminant analysis – Standardized canonical discriminant function coefficients for males morphometric characteristics for each discriminant function. Also eigenvalues, percentage of explained variance (% Expl. var.), cumulative proportions (Cum. prop.) and canonical correlations (Canonical R) are given.

Characteristic	Function 1	Function 2
CLW	-2.520	-1.246
CPH	0.248	-0.945
CFL	2.039	-0.851
ROW	-0.703	-0.742
ABW	0.426	-0.666
CLH	0.756	-0.391
TL	-0.319	-0.246
ARW	-0.742	-0.034
ARL	-0.293	0.290
CGW	-0.379	0.375
ROL	1.739	0.449
CEW	-0.359	0.611
TEW	-0.628	0.651
CPL	1.686	0.727
ABH	-0.236	0.739
CLL	-1.406	1.610
Eigenvalue	6.246	1.184
% Expl. var.	84.010	15.990
Cum. Prop.	0.841	1.000
Canonical R	0.928	0.736

Table 4 Results of discriminant analysis – Standardized canonical discriminant function coefficients for females morphometrical characteristics for each discriminant function. Also eigenvalues, percentage of explained variance (% Expl. var.), cumulative proportions (Cum. prop.) and canonical correlations (Canonical R) are given

Characteristic	Function 1	Function 2
TL	2.283	-1.793
CEW	0.119	-1.759
CEF	0.069	-1.064
ABH	-0.250	-0.853
TEW	-0.987	-0.366
ROL	0.955	-0.290
ALFA	-2.670	-0.222
ARW	-1.809	0.162
ROW	0.116	0.651
ARL	-0.500	0.654
ABW	0.217	1.155
CPH	1.960	1.555
CPW	-5.290	2.940
Eigenvalue	5.249	1.376
% Expl. var.	79.230	20.770
Cum. Prop.	0.792	1.000
Canonical R	0.916	0.761

Table 5 Percentages of correctly classified crayfish based on the function of the corresponding discriminant analyses for both males (% males) and females (% females).

Location / population	% males	% females
Blate	100	100
Zeleni vir	100	92.31
Sopotski slap	90.91	92.31
Total	98.18	96.49

Figure legends

Fig. 1 Position of the Nature Park Žumberak-Samoborsko gorje within north-west Croatia with distribution of stone crayfish phylogroups (Klobučar et al., 2013) in Europe ("Zeleni Vir" - pinpoint, "Gorski Kotar" - triangle, "Žumberak, Plitvice and Bjelolasica" - star, "Lika and Dalmatia" - cross, "Banovina" - diamante, "southern Balkan" - square, "central and south-eastern Europe" - circle. On the smaller left map position of the studied stone crayfish populations within the borders of Nature Park Žumberak-Samoborsko gorje is presented (Zeleni Vir - pinpoint, Blate - triangle, Sopotski slap - star).

Fig. 2 Discrimination of the different populations of *Austropotamobius torrentium* males by the first two discriminant functions

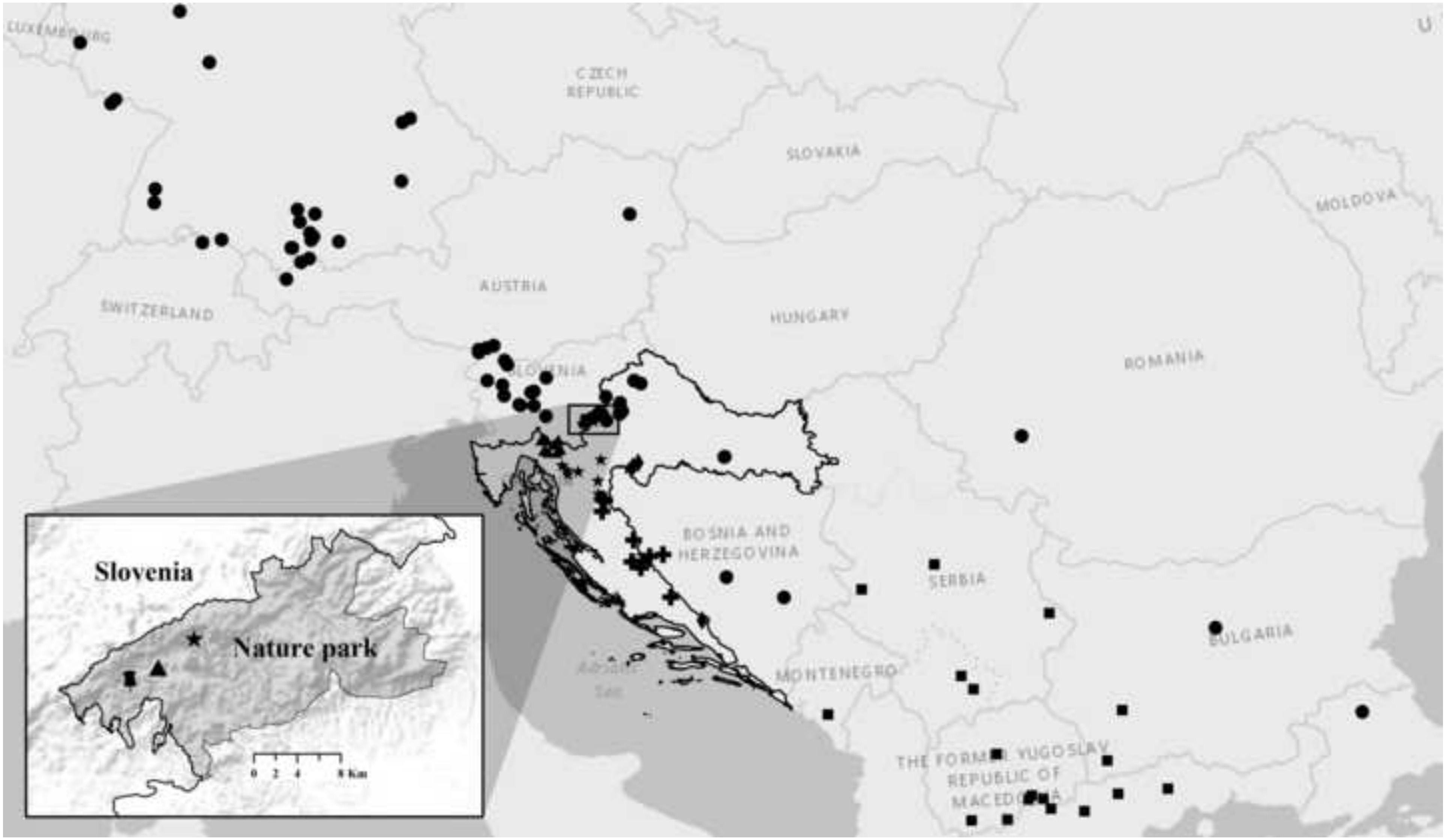
Fig. 3 Discrimination of the different populations of *Austropotamobius torrentium* females by the first two discriminant functions

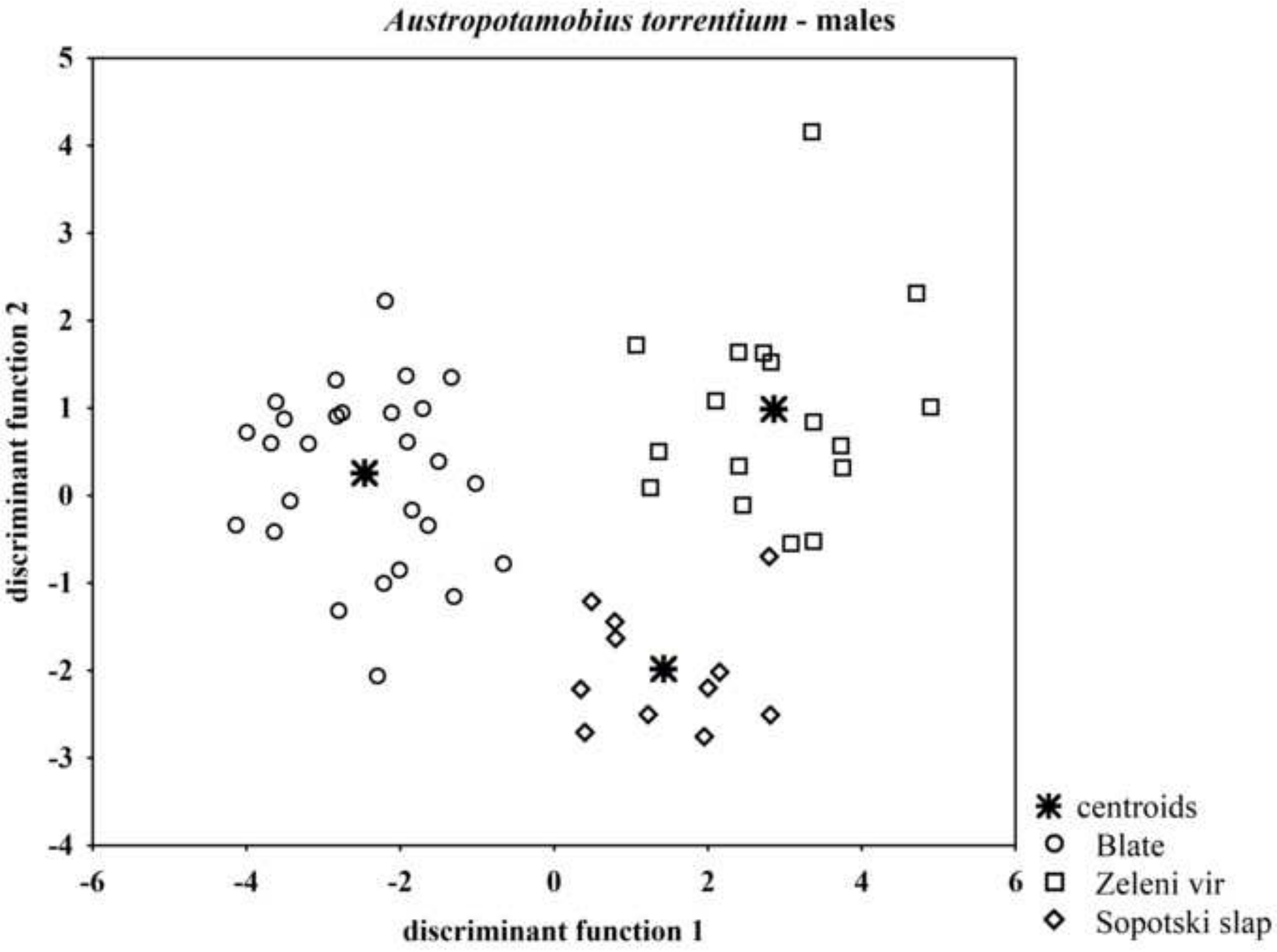
Fig. 4 Mean number of spines on the merus of the third maxilliped recorded per population. Asterisks or hash denote statistically significant differences between populations

Fig. 5 Percentage of different type of denticulation (spines or tubercles) on the lower surface of antennal exopod per population

Fig. 6 Percentage of different pronunciation (weak, strong) of median rostral carina per population

Figure1


LUXEMBOURG
CZECH REPUBLIC
SLOVAKIA
MOLDOVA
SWITZERLAND
AUSTRIA
HUNGARY
SLOVENIA
ROMANIA
BOSNIA AND HERZEGOVINA
SERBIA
BULGARIA
Adriatic Sea
MONTENEGRO
THE FORMER YUGOSLAV REPUBLIC OF MACEDONIA
Slovenia
Nature park
0 2 4 8 Km


Austropotamobius torrentium - males
discriminant function 2
5
4
3
2
1
0
-1
-2
-3
-4
-6
-4
-2
0
2
4
6
discriminant function 1
centroids
Blate
Zeleni vir
Sopotski slap

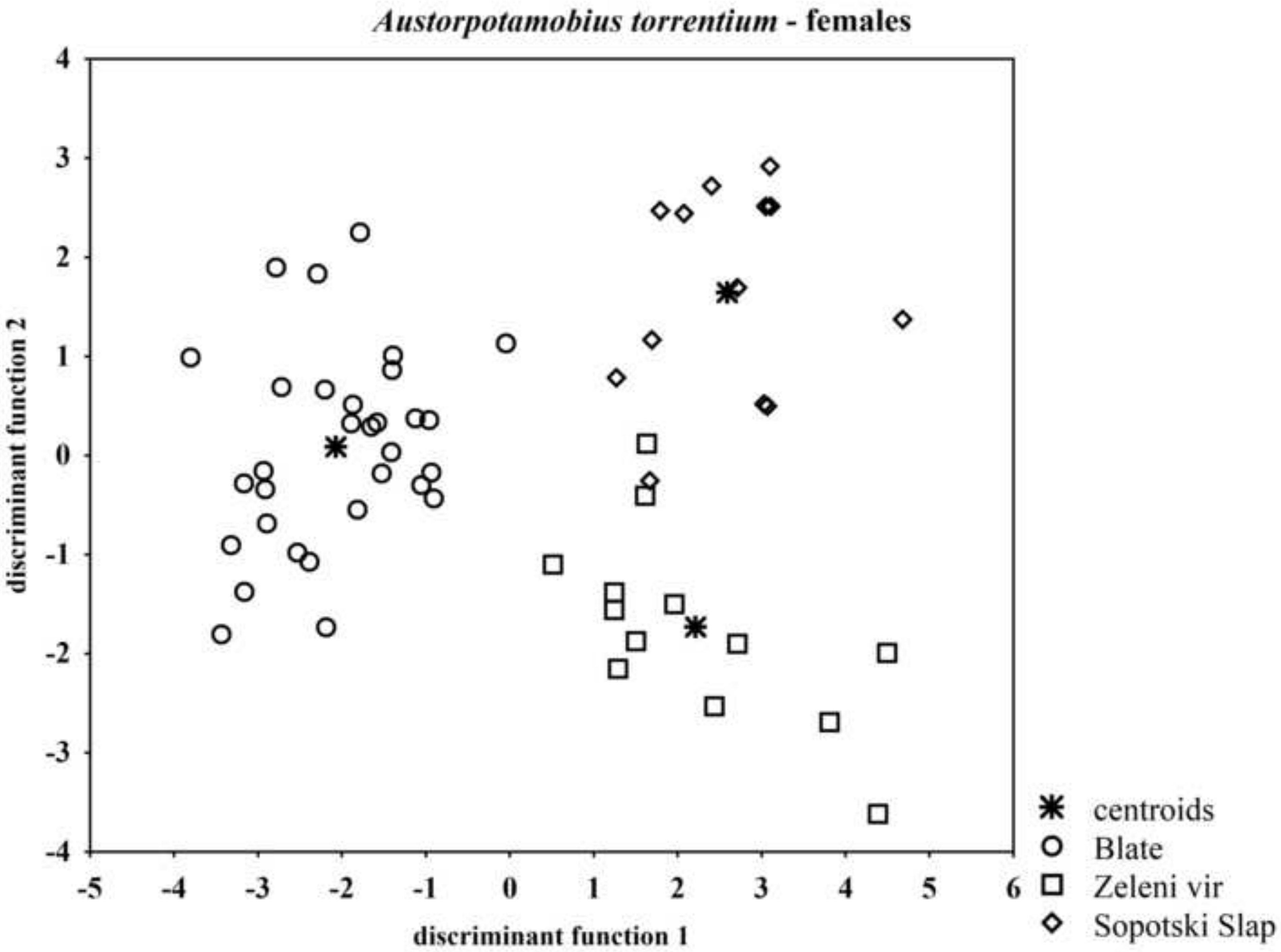
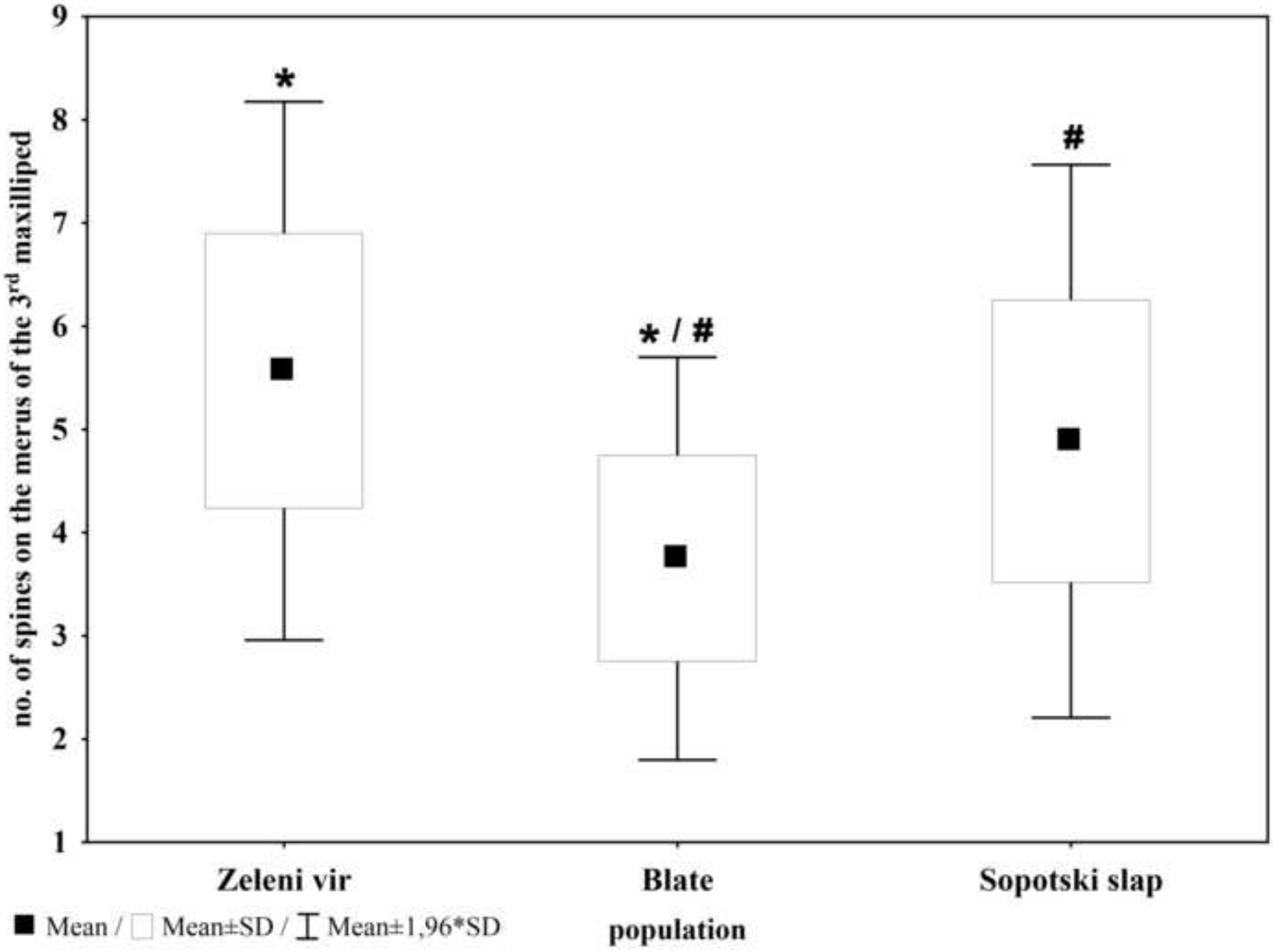
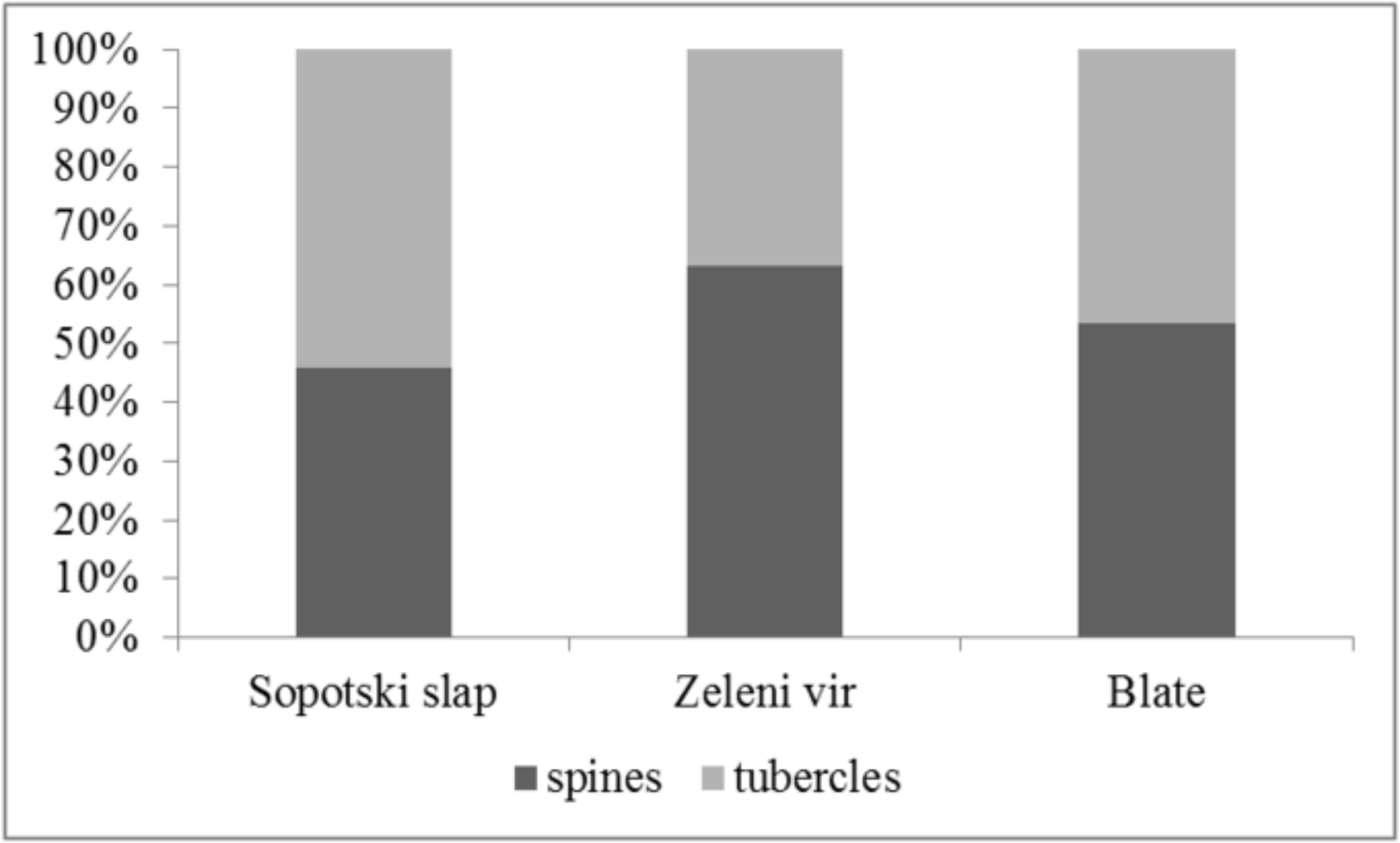
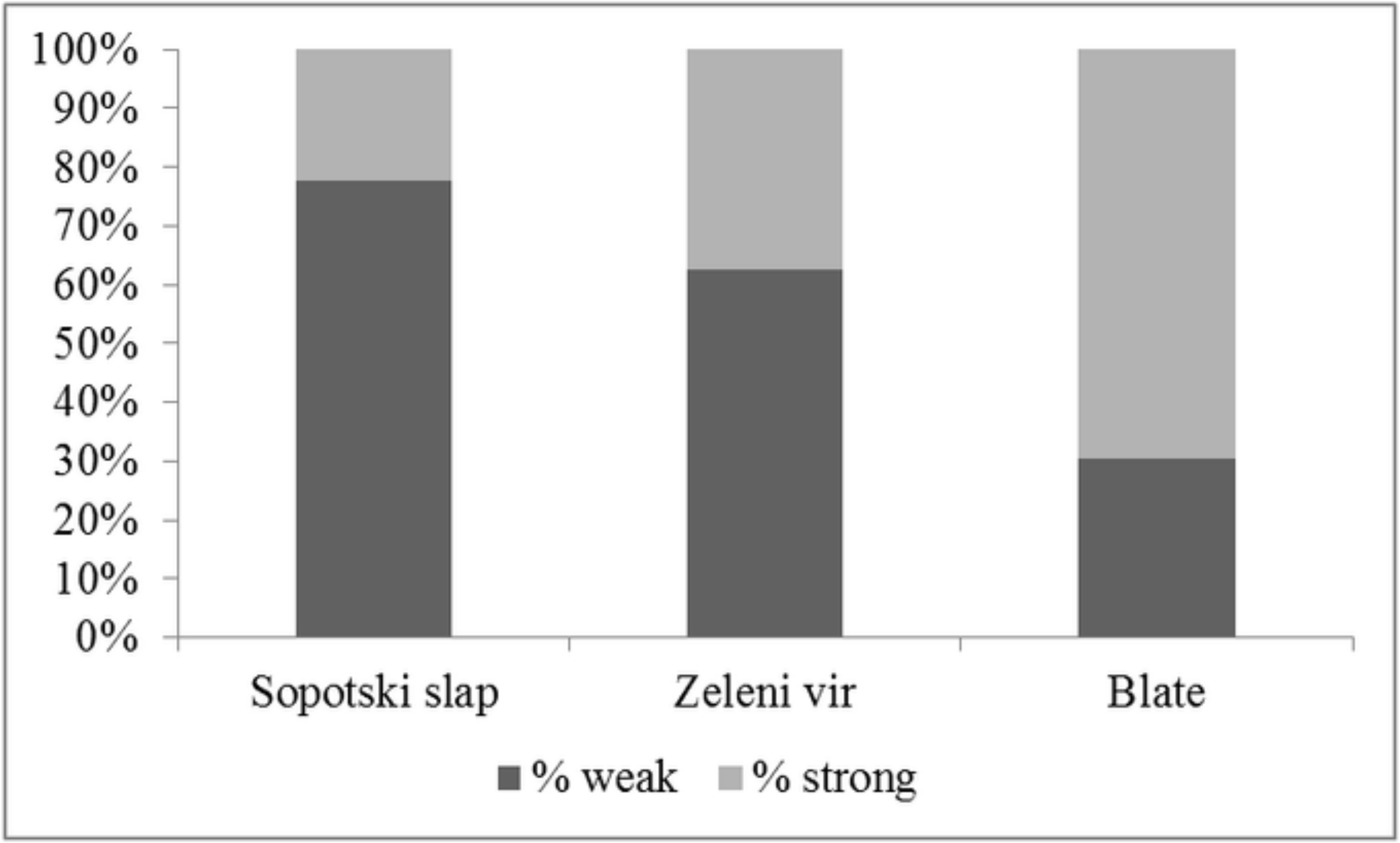

Austorpotamobius torrentium - females
discriminant function 2
discriminant function 1
4
3
2
1
0
-1
-2
-3
-4
-5
-4
-3
-2
-1
0
1
2
3
4
5
6
✱ centroids
○ Blate
□ Zeleni vir
◇ Sopotski Slap

Figure4


no. of spines on the merus of the 3rd maxilliped
9
8
7
6
5
4
3
2
1
*
* / #
#
Zeleni vir
Blate
Sopotski slap
population
■ Mean / ☐ Mean±SD / Ⅰ Mean±1,96*SD


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Sopotski slap
Zeleni vir
Blate
spines
tubercles


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Sopotski slap
Zeleni vir
Blate
■ % weak
■ % strong