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Morphological diversity of the stone crayfish − traditional and geometric morphometric approach

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Abstract – *Austropotamobius torrentium* is one of four native European crayfish species inhabiting Croatian freshwaters. Existence of eight divergent monophyletic mtDNA phylogroups was described within *A. torrentium*; six of them are distributed in Croatia, with the highest genetic diversity established in its northern-central Dinaric region. Recent small-scale study of the stone crayfish morphological variability indicated significant differences among different phylogroups. In the present study larger sample size, covering populations from five phylogroups, was analysed with the aim of determining whether there are morphological characteristics that reliably separate stone crayfish from different phylogroups. Aiming this, 245 stone crayfish were analysed through traditional (TM) and, for the first time, geometric morphometric (GM) analyses. Multivariate discriminant analyses included 24 TM characteristics per crayfish, while GM comprised analyses of 22 landmarks on the dorsal side of cephalon. Both methods revealed congruent results, and significant differences among phylogroups in analysed features were obtained, with the cephalon shape contributing the most to crayfish discrimination. Research confirmed that both approaches, combined with statistical methods, are useful in distinguishing and separating crayfish phylogroups. Findings of present study are compatible with the previous molecular findings; stone crayfish present several distinct evolutionary lineages whose species status are currently undefined and require urgent clarification.

Keywords: *Austropotamobius torrentium* / Generalized Procrustes Analysis / landmark / multivariate discriminant analysis / semilandmark

Résumé – Diversité morphologique de l'écrevisse des torrents − approche morphométrique traditionnelle et géométrique. *Austropotamobius torrentium* est l'une des quatre espèces indigènes d'écrevisses européennes qui peuplent les eaux douces croates. L'existence de huit phylogroupes divergents d'ADNmt monophylétiques a été décrite chez *A. torrentium* ; six d'entre eux sont distribués en Croatie, avec la plus grande diversité génétique établie dans sa région dinarique nord-centre. Une étude récente à petite échelle de la variabilité morphologique de l'écrevisse des torrents a révélé des différences significatives entre les différents phylogroupes. Dans la présente étude, on a analysé des échantillons de plus grande taille, couvrant des populations de cinq phylogroupes, dans le but de déterminer s'il existe des caractéristiques morphologiques qui séparent de façon fiable les écrevisses des torrents des différents phylogroupes. Dans ce but, 245 écrevisses des torrents ont été analysées par des analyses traditionnelles (TM) et, pour la première fois, morphométriques géométriques (GM). Les analyses discriminantes à plusieurs variables comprenaient 24 caractéristiques TM par écrevisse, tandis que les analyses GM comprenaient des analyses de 22 repères sur la face dorsale du céphalon. Les deux méthodes ont révélé des résultats congruents, et des différences significatives entre les phylogroupes dans les traits analysés ont été obtenues, la forme du céphalon contribuant le plus à la discrimination des écrevisses. La recherche a confirmé que les deux approches, combinées à des méthodes statistiques, sont utiles pour distinguer et séparer les phylogroupes d'écrevisses. Les résultats de la présente étude sont compatibles avec ceux des études moléculaires précédentes ; les écrevisses des torrents présentent

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plusieurs lignées évolutives distinctes dont le statut d'espèce n'est pas encore défini et nécessite une clarification urgente.

Mots-clés : *Austropotamobius torrentium* / Analyse procrustéenne généralisée / points de repère / analyse discriminante multivariée / marques de semi-marques

1 Introduction

The stone crayfish *Austropotamobius torrentium* (Schrank, 1803) is one of four native European crayfish species inhabiting streams and smaller rivers at higher altitudes of both the Black Sea and the Adriatic Sea basins in Croatian freshwater ecosystems (Maguire *et al.*, 2011). In the last few decades, noticeable declines of stone crayfish populations have been recorded across its distribution range in Europe (Weinländer and Füreder, 2009; Kouba *et al.*, 2014). In Croatia, losses are mainly caused by anthropogenic pressure onto the stone crayfish habitats (fragmentation, waterbody engineering), presence and spread of non-native invasive crayfish species and diseases they transmit (*e.g.* crayfish plague), as well as climate changes (Maguire *et al.*, 2018). Therefore, the stone crayfish is considered a threatened species, protected by national (NN 80/13, 144/13) and international legislation (Appendix III of the Bern Convention, Appendices II and V of the Habitat Directive (92/43/EEC and 97/62/EU)). Efficient protection of existing populations requires development of conservation and management plans based on the sound knowledge of species, including information on genetics and morphology (Peay, 2009; Souty-Grosset and Reynolds, 2009).

Previous molecular-phylogenetic research based on mtDNA by Trontelj *et al.* (2005) indicated that the stone crayfish should be considered a species complex. Later, Klobučar *et al.* (2013) discovered the existence of at least seven divergent monophyletic phylogroups within *A. torrentium*, with the highest genetic diversity recorded in the Dinaric region of Croatia, while recently Pârvulescu *et al.* (2019) described another phylogroup endemic to the Romanian Apuseni region. Moreover, latest analyses of meristic and morphometric features of several stone crayfish populations, belonging to different phylogroups, indicated their significant differences and point to necessity of additional analyses that would cover bigger sample size and wider area (Maguire *et al.*, 2017). Following that idea, a new *Austropotamobius* crayfish species was just described (Pârvulescu *et al.*, 2019).

In this study we included larger sample size, complementing data set from Maguire *et al.* (2017) covering populations from five out of seven previously described mtDNA phylogroups (Klobučar *et al.*, 2013). Also, apart from traditional morphometry, we used, for the first time geometric morphometrics on *A. torrentium*. Geometric morphometrics (GM) is a method with a growing application in different organisms' studies (Caumul and Polly, 2005; Cardini *et al.*, 2007; Becking *et al.*, 2016). It proved itself as a powerful tool in detecting differences in morphology within and among species (Lawing and Polly, 2010). Up till now, in the freshwater crayfish research, GM has been applied in the studies on the white-clawed crayfish (*Austropotamobius pallipes* (Lereboullet, 1858)) (Scalici *et al.*, 2010; Scalici and Bravi, 2012), red swamp crayfish (*Procambarus clarkii* (Girard, 1852)) (Malavé *et al.*, 2018), rusty crayfish (Perry *et al.*, 2013) and two *Cambarus* species (*Cambarus halli*

Hobbs, 1968 and *Cambarus englishi* Hobbs & Hall, 1972) (Helms *et al.*, 2015).

The aim of this study was to verify if there are significant differences among stone crayfish belonging to different phylogroups, as previous preliminary results indicated (Maguire *et al.*, 2017). Further, the intention was to find which traditional morphometric features discriminate the best different phylogroups. Another objective was to validate if landmark-based GM analyses are suitable for detecting differences in shape among different *A. torrentium* phylogroups. Finally, the goal was to verify if results of the two methods (traditional and geometric morphometrics) yield congruent results. We expected that morphology of stone crayfish differs among different phylogroups and that results obtained by the two methods are congruent and compatible with the previous molecular findings.

2 Materials and methods

2.1 Sampling

Prior to field work, all of the required permits (working in protected areas, studying strictly protected species) were obtained from the Ministry of Environmental Protection and Energy of the Republic of Croatia. Crayfish were collected from 15 localities covering populations from five previously described mtDNA phylogroups: Gorski Kotar (GK); Žumberak, Plitvice and Bjelolasica (ŽPB); Lika and Dalmatia (LD), Banovina (BAN) and central and south-eastern Europe (CSE) (Trontelj *et al.*, 2005; Klobučar *et al.*, 2013) (Tab. 1, Fig. 1). Sampling was performed by hand during the night or with hand-made traps (Maguire *et al.*, 2002) placed along banks of the stream and left in the water overnight. All captured crayfish specimens were identified to the species level and their sex was determined. Only adult (total length > 5 cm (Huber and Schubart, 2005; Maguire and Klobučar, 2011)), uninjured, intermolt crayfish were examined and measured; 245 individuals were included into traditional morphometric (TM) analyses, and out of them 209 into geometric morphometric (GM) analyses. Discrepancy in the number is due to the fact that some of the landmark positions on some of the crayfish were indistinct, so those crayfish were omitted from the GM analyses.

2.2 Traditional morphometric analyses

In order to perform traditional morphometric analyses, we measured 24 morphometric characteristics per each crayfish, using a digital calliper with precision of 0.01 mm; 21 were taken from Sint *et al.* (2005); claw width (CLW), claw length (CLL), claw height (CLH), length of the claw finger (CFL), length of the claw palm (CPL), rostrum width (ROW), rostrum length (ROL), head width (HEW), head length (HEL), areolar width (ARW), areolar length (ARL), abdomen width (ABW), abdomen height (ABH), abdomen length (ABL), telson width

Table 1. List of samples; mtDNA phylogroups *sensu* Klobučar *et al.* (2013); (Banovina (BAN), Lika and Dalmatia (LD), Gorski Kotar (GK), Žumberak, Plitvice and Bjelolasica (ŽPB), and central and south-eastern Europe (CSE)) with sampled localities (streams) and number of stone crayfish per group (total, and number of males and females).

mtDNA phylogroup (locations)	Crayfish number (Male/Female)
BAN (Bručina, Maja)	42 (16/26)
LD (Krasulja, Orašnica)	61 (45/16)
GK (Bresni, Delnički, Vele vode)	30 (20/10)
ŽPB (Slapnica, Jarak, Sopotski slap, Sartuk)	33 (31/2)
CSE (Bliznec, Dolje, Okičnica, Jarak-Stojdraga)	79 (40/39)

(TEW), telson length (TEL), width of the carapace at the hind edges (CEW), carapace height (CPH), carapace width (CPW), width at the cervical groove (CGW), and total length (TL); and extra three included cephalothorax length (CEF), apex length and width (APL and APW, respectively). Bilateral characteristics were recorded on the right body side because it was proven that bilateral characteristics are symmetrical and show no differences between the two body sides (Maguire *et al.*, 2017). Further, all of the measured morphometric characteristics were normalised by dividing their value with the corresponding postorbital length (POL = HEL + ARL) to avoid comparison of different sized crayfish that could lead to false results (Chambers *et al.*, 1979; Palma and Andrade, 2002; Sint *et al.*, 2005). Since *t*-test showed that males and females differed significantly in characteristics describing claws and abdomen, the two sexes were analysed separately (Sint *et al.*, 2007; Berger *et al.*, 2017; Maguire *et al.*, 2017; Vlach and Valdmanová, 2015). Observed differences are consequence of sexual dimorphism that is characteristic of crayfish and appears upon crayfish attein sexual maturity (Grandjean *et al.*, 1997; Streissl and Hödl, 2002; Vlach and Valdmanová, 2015). Analyses were performed in Microsoft Excel (version 2010) and Statistica 13 (StatSoft. Inc.). ANOVA with Bonferroni post-hoc test was applied to verify if there were differences among phylogroups in the measured morphometric characteristics. The multivariate discriminant analysis (MDA) was conducted in order to analyse differentiation of phylogroups based on their morphometric characteristics, and to select morphometric characteristics that made the most significant contribution to separation of crayfish belonging to different phylogroups. The results of the canonical discriminant analysis were visualised by scatterplots for the two discriminant functions.

2.3 Geometric morphometric analyses

Geometric morphometric (GM) method was applied for a detailed analysis of shape variations of crayfish cephalon, especially focussing on the shape of the rostrum and apex. Data for both sexes were merged because no significant differences were found between the two sexes in log-transformed centroid sizes ($t = 0.416$; $p = 0.677$) what is in accordance with previous GM studies by Scalici *et al.* (2010)
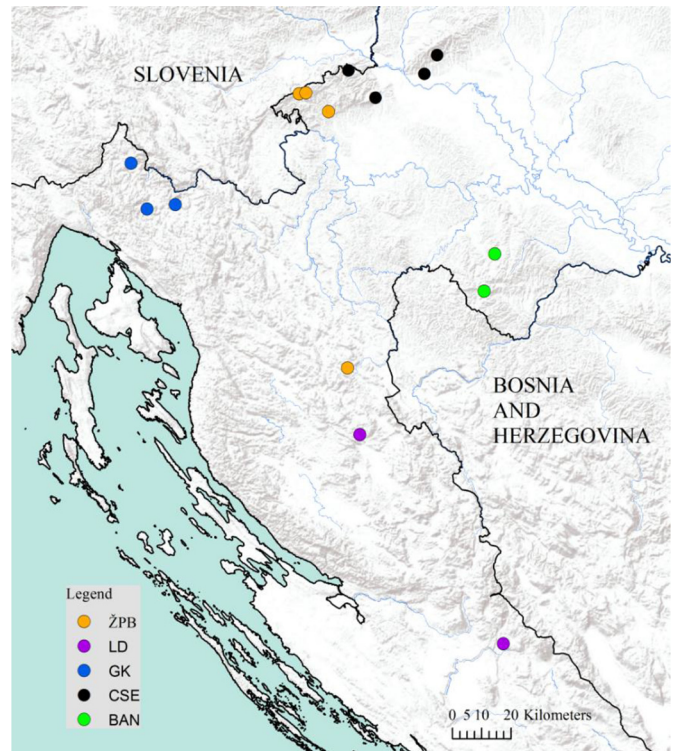

Fig. 1. Map of the study area with sampling sites produced in ArcGIS 10.3 program package. Different colours represent different phylogroups *sensu* Klobučar *et al.* (2013); ŽPB − Žumberak, Plitvice and Bjelolasica; LD − Lika and Dalmacija; GK − Gorski Kotar; CSE − Central and south-eastern Europe; BAN − Banovina

on *A. pallipes* (*A. torrentium* sister species) which revealed lack of sexual dimorphism in GM variables. In order to perform landmark-based morphometrics analyses, a digital picture of the dorsal side of each crayfish cephalon was obtained by scanning cephalon portion of each animal on the Epson Perfection V600 Photo scanner. Each specimen was positioned with dorsal side downwards in a water basin placed on flatbed scanner, while the area of interest (cephalon) was kept parallel to scanner by hand. Images were scaled to cm scale. GM analyses were performed using TPS series (Rohlf, 2015) and MorphoJ (Klingenberg, 2011). The location of 22 equally distributed two-dimensional specific measuring points (12 landmarks − LM and 10 semilandmarks − SM) on the dorsal side of the crayfish cephalon were digitised using software TpsDig2 (Rohlf, 2015) (Fig. 2). Position of LMs and SMs was modified according to Scalici *et al.* (2010) and Scalici and Bravi (2012). Semilandmarks were used to describe variation in the shape of rostrum apex and were treated as equivalent to landmarks when computing superimposition. They were defined as equally distant points between corresponding landmarks as described in Zelditch *et al.* (2004). Digitalisation of the LM and SM was made in the same order on each picture, after setting a scale factor. Each specimen was subjected to Generalized Procrustes Analysis (GPA), a procedure that separates the form of an organism into two components, centroid size and shape, by eliminating the non-shape variation resulting from positioning, orientation and
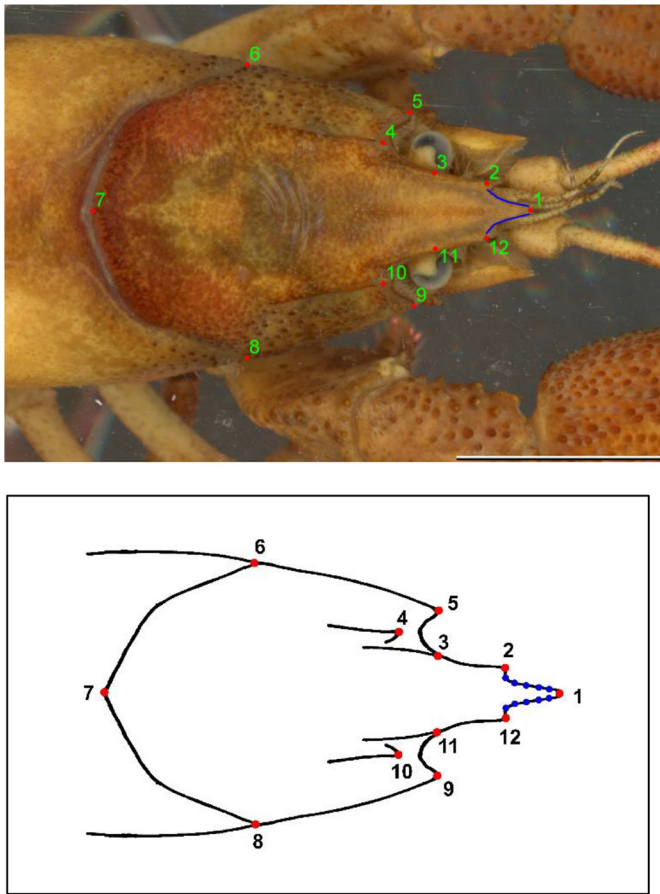
Fig. 2. Position of the landmarks and semilandmarks on the dorsal side of stone crayfish. Above − original scan with 12 landmarks (red dots) and 10 semilandmarks (blue lines); Under − drawing 12 landmarks (red dots) and 10 semilandmarks (blue dots)

Table 2. Standardized canonical discriminant function coefficients for morphometric characteristics of *A. torrentium* males classified in five phylogroups (LD, GK, ŽPB, BAN and CSE) *sensu* Klobučar *et al.* (2013); for each discriminant function (root1-root4).

Characteristic	Root 1	Root 2	Root 3	Root 4
CLH-d	−0.414	2.313	0.067	−1.273
CPL-d	1.616	−0.212	−0.617	−1.133
TEL	−0.477	−0.183	−0.207	−0.849
ABL	−0.013	0.255	0.759	−0.359
CEF-d	−0.018	−0.215	0.546	−0.304
CGW	−0.743	0.276	0.270	−0.106
ABH	0.185	0.025	0.296	0.143
CFL-d	−0.278	0.201	0.161	0.148
CPH	0.227	0.106	0.179	0.156
HEW	0.475	−0.181	0.061	0.189
TEW	0.265	0.036	−0.785	0.247
ABW	−0.071	−0.193	0.158	0.250
HEL	0.083	0.049	0.283	0.266
ARW	0.271	−0.237	−0.490	0.280
CPW	0.3291	−0.128	−0.846	0.283
ROW	−0.6921	0.073	0.019	0.342
CLW-d	−0.3221	0.169	−0.628	0.427
APW	0.489	0.362	0.017	0.550
CLL-d	−0.801	−2.655	0.672	1.838
Eigenvalue	10.587	1.208	0.718	0.183
Cum. Prop.	0.834	0.929	0.985	1.000
% Expl. Var.	83.381	92.902	98.557	100.000
Canonical R	0.955	0.739	0.646	0.393

scaling (Rohlf and Slice, 1990; Bookstein, 1991). Procrustes coordinates represent shape variables, while centroid size represents size variable independent of shape; it equals the square root of the sum of squared distances of each landmark from their centroid (Zelditch *et al.*, 2004). The measurement error is important when analysing shape in GM, due to possible errors in the landmark and semilandmark digitisation (Klingenberg and McIntyre, 1998; Klingenberg *et al.*, 2002). In order to evaluate the significance of measurement error, a sample of 50 randomly picked individuals was digitised twice by the same operator. Procrustes ANOVA was conducted to access the measurement error by comparing the mean square values of the individual variation with the mean square values of the digitised Error 1.

The patterns of shape variation among five *A. torrentium* phylogroups were examined by carrying out a canonical variate analysis (CVA). Since our aim was to analyse if stone crayfish from different phylogroups differ significantly in the shape, we chose performing CVA over PCA (principle component analysis). It is recommended to use PCA when variation among individuals is analysed, whereas CVA is suggested to be used for describing differences between groups (Zelditch *et al.*, 2004). Variation and changes in shape described by CVA were graphically depicted in the form of

wireframe to visualise the differentiation of the crayfish belonging to different phylogroups.

In order to establish correlation between TM and GM distances, Mantel test was conducted (Mantel, 1967) in XLSTAT ver. 2019, implemented in Excel, using 9999 permutations. For TM distance matrix, Euclidian distances for measured morphometric characteristics among phylogroups were calculated, while for GM distance matrix we used Procrustes distances among phylogroups.

A level of significance of 1% ($P < 0.01$) was used in all statistical analyses.

3 Results

3.1 Traditional morphometry

Traditional morphometry (TM) analyses included 245 crayfish from 15 populations assigned to five phylogroups, of which 89 were females and 156 males. Results of ANOVA displayed significant differences between phylogroups in the measured morphometric characteristics, for both males ($F = 4.01$; $P < 0.01$) and females ($F = 3.35$; $P < 0.01$) (details are not shown), therefore we proceeded with multivariate discriminant analysis. MDA revealed that morphometric characteristics with the highest discriminatory impact (highest loadings in discriminant functions) for males were those describing claws (CLL, CPL, CLH) and head region (ROW, CGW) (Tab. 2), while for females they included claws (CLL, CLH), head (ROL, HEL and APW) and abdomen (TEW, ABW

Table 3. Standardized canonical discriminant function coefficients for morphometric characteristics of *A. torrentium* females classified in five phylogroups (LD, GK, ŽPB, BAN and CSE) *sensu* Klobučar *et al.* (2013); for each discriminant function (root1-root4).

Characteristic	Root 1	Root 2	Root 3	Root 4
CLL-d	–0.698	–0.398	1.506	–1.117
TEW	–0.527	0.348	0.567	0.038
ARW	–0.486	0.129	0.328	–0.398
APW	–0.465	–0.408	–0.281	0.791
ABH	–0.433	–0.015	0.037	–0.042
HEW	–0.323	0.069	–0.351	–0.190
ABW	–0.246	–0.515	0.756	0.341
CPW	–0.196	–0.328	0.333	0.155
HEL	–0.127	–0.644	0.059	0.062
CFL-d	–0.018	–0.154	–0.576	0.492
TL	0.059	–0.138	–1.746	0.586
ABL	0.187	0.465	0.101	–0.420
CLH-d	0.199	0.564	–0.866	0.237
CLW-d	0.218	–0.126	–0.176	0.488
CEW	0.242	0.317	–0.610	–0.538
CGW	0.251	–0.406	0.129	0.282
TEL	0.518	0.526	0.287	0.546
CPH	0.576	0.156	–0.255	0.265
ROL	1.132	–0.156	0.344	0.026
Eigenvalue	5.316	1.399	0.978	0.635
Cum.Prop	0.638	0.806	0.923	1.000
% expl. Var.	63.820	80.625	92.370	99.993
Canonical R	0.917	0.763	0.703	0.623

Table 4. Percentages of correctly classified individuals of *A. torrentium* based on the function of the corresponding discriminant analyses for males (% males) and females (% females); Lika and Dalmatia (LD), Gorski Kotar (GK), Žumberak, Plitvice and Bjelolasica (ŽPB), Banovina (BAN) and central and south-eastern Europe (CSE).

Phylogroup	% males	% females
LD	95.55	100.00
GK	95.00	90.00
ŽPB	93.55	50.00
BAN	58.82	80.00
CSE	90.90	85.71
Total	89.80	86.36

Fig. 3. Discrimination of the different phylogroups of a) males of *A. torrentium* by the two discriminant function (Root 1/Root 2) and b) females of *A. torrentium* by the first and third discriminant functions (Root 1/Root 3). Different colour dots represent different phylogroups: green dots − Banovina (BAN), black dots − central and south-eastern Europe (CSE), blue dots − Gorski Kotar (GK), purple dots − Lika and Dalmatia (LD), orange dots − Žumberak, Plitvice and Bjelolasica (ŽPB)

and CEW) (Tab. 3). Stepwise analysis revealed high number of correctly classified males and females per phylogroups: 89.81% of males and 86.36% of females were classified correctly (Tab. 4).

Canonical variate analyses were performed on populations classified in five *a priori* defined phylogroups: LD, GK, ŽPB, BAN and CSE. Scatter plots for the discriminant functions (roots) are shown in Figure 3a (males) and b (females). The first discriminant function discriminated well males belonging to t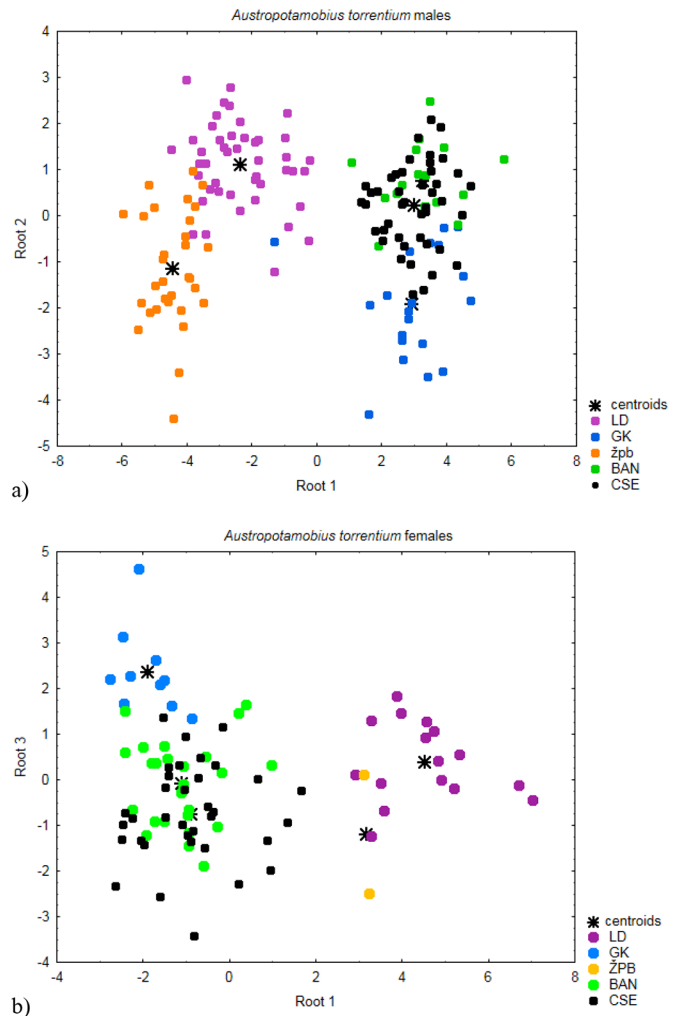he phylogroups ŽPB and LD from males belonging to the phylogroups GK, CSE and BAN (Fig. 3a). The first discriminant function was marked with high negative loadings for CLL, CGW and ROW and with high positive loadings for CPL and APW, therefore the smaller the values of CLL and ROW, the more likely males belong to the phylogroups ŽPB or LD, and the higher the values of CPL and APW, the more likely males belong to the phylogroups GK, CSE and/or BAN. The second discriminant functions discriminated to some extant males from the phylogroups ŽPB and GK from the rest. Since second discriminant function was marked with high negative loadings for CLL and high positive loadings for CLH and APW, we may assume the smaller the values of CLL, the more likely males belong to the phylogroups ŽPB or GK, and the higher the values of CLH and APW, the more likely males belong to the phylogroups LD, CSE and/or BAN.

Table 5. Measurement error assessed by performing Procrustes ANOVA on centroid size and shape of stone crayfish. Sums of squares (SS) and mean squares (MS) are in units of Procrustes distances.

Effect	SS	MS	df	F	*P* (param.)	Pillai tr.	*P* (param.)
Centroid size							
Individual	0.000038	0.000001	49	2.03	0.007		
Error 1	0.000019	0.000000	50				
Shape							
Individual	0.18727328	0.0001910952	980	3.07	<.0001	14.02	<.0001
Error 1	0.02474525	0.0000123726	2000				

For females, the first discriminant function (root1) discriminated individuals belonging to the phylogroup LD and ŽPB from those belonging to GK, BAN and CSE (Fig. 3b). The first discriminant function was marked with high negative loadings for CLL and TEW or ARW and with high positive loadings for ROL and CPH. Accordingly, it can be presumed the smaller the values of CLL and TEW or ARW the more likely females belong to the phylogroups CSE, BAN and/or GK, and the higher the values of ROL and CPH, the more likely females belong to the phylogroups LD and/or ŽPB. The third discriminant function (root3) discriminated females belonging to phylogroups GK from the rest. Since the third discriminant function was marked with high negative loadings for TL and CLH and with high positive loadings for CLL and ABW, we may say the smaller the values of TL and CLH the less likely females belong to the phylogroup GK, while the higher the values of CLL and ABW the more likely females belong to the phylogroup GK.

3.2 Geometric morphometrics

Geometric morphometric analyses included 209 crayfish from 15 populations belonging to five previously described mtDNA phylogroups. Generalized Procrustes Analysis revealed the centroid size as size variables and Procrustes coordinates as shape variables. The Procrustes ANOVA was applied to assess the measurement error, and results showed that the mean square for individual variation exceeded the measurement error; therefore it was negligible (Tab. 5).

Results of CVA showed the differentiation among five *A. torrentium* phylogroups caused by the cephalon shape variation (Fig. 4). The first two canonical variates (CV1 and CV2) explained 71.41% of the total variation of the cephalon shape; CV1 accounted for 45.95% of the variability while the CV2 accounted for 25.46% of the variability. The CV1 and CV2 mostly separated phylogroups ŽPB, GK, LD, while there was an overlapping between the phylogroups BAN and CSE. The cephalon shape, as quantified by Procrustes distances, differed between the phylogroups (Tab. 6). The highest values of the Procrustes distances were obtained between the phylogroups ŽPB and BAN (0.0599), followed by ŽPB and CSE (0.0548), while the lowest values of Procrustes distances were observed between CSE and BAN (0.0210), and CSE and LD (0.0269). All Procrustes distances between the phylogroups were statistically significant with p-values lower than 0.01.

Morphological variability of cephalon was mainly visible in the anterior part, while the posterior area was less variable in the shape (Fig. 4). Shape changes that contribute to the distinction among crayfish from different phylogroups were visible in the apical part of the cephalon, particularly in the rostrum apex, rostrum base and width of the lateral edge of the head. Shape changes along +CV1 are characterised by general widening of the rostrum, shortening (LM 7) and narrowing (LM 6 and 8) of the head, shifting of the lateral edge of the head towards its apical part (LM 5 and 9), and elongation of the apex (SM 1-2 and 1-12). These characteristics were present in the majority of the specimens belonging to the phylogroup GK, CSE, BAN and partly ŽPB. In contrast, −CV1 was related to reduction of rostrum size and length, elongation (LM 7) and widening (LM 6 and 8) of the head, shifting of the lateral edge of the head towards its distal part (LM 5 and 9), and shortening of the apex (SM 1-2 and 1-12). These morphological characteristics were generally present in the phylogroup LD and partly in the phylogroup ŽPB. Shape changes along +CV2 were characterised by larger head, narrower elongated rostrum (LM 2-3 and 11-12 and SM 13-17 and 18-22), and longer apex (SM 1-2 and 1-12). Shape changes along −CV2 were characterised by wider and shorter rostrum (LM 2- 3 and 11-12; SM 13-17 and 18-22), less robust head and shorter apex (SM 1-2 and 1-12). These morphological characteristics were generally pronounced in the crayfish from the phylogroup ŽPB, GK and partly LD.

The Mantel test showed significant correlation between TM and GM distance matrices ($r = 0.756$, $p = 0.007$).

4 Discussion

This study aimed to determine morphological characteristics which separate stone crayfish belonging to different mtDNA phylogroups through analyses of traditional morphometric features, and for this species for the first time through geometric morphometric approach. Also, the goal was to verify if the two methods yield congruent results, and if results of morphometry are consistent with the results obtained in previous molecular research (Klobučar *et al.*, 2013). Overall, results of morphometry were in agreement with results obtained in previous molecular research (Klobučar *et al.*, 2013) and established significant differences in morphology among stone crayfish from different phylogroups, with characteristics of cephalon (both TM and GM) and claws (TM) contributed the most to their divergence.
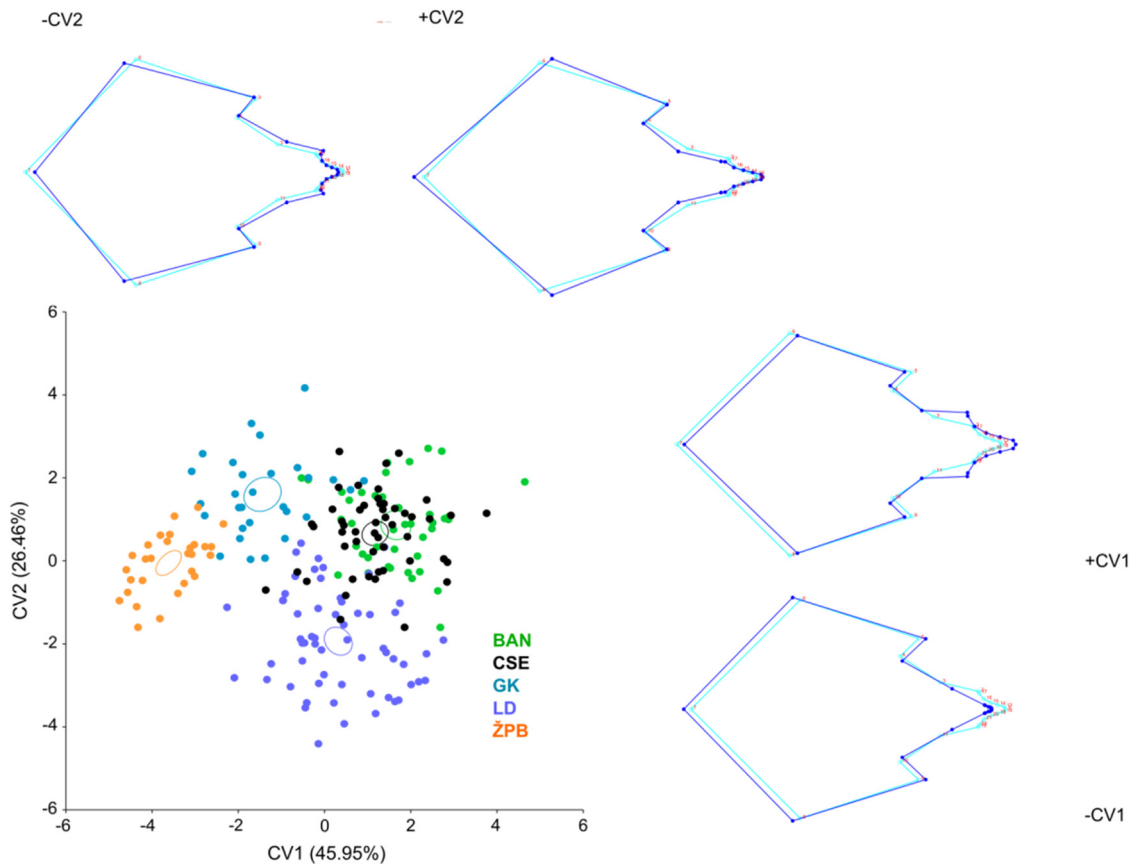
Fig. 4. Cephalon shape variation among the phylogroups of *A. torrentium* revealed by canonical variate analysis. Scatter plott of the two first canonical variate axes (CV1 and CV2) with associated wireframe representation depicting shape changes among phylogroups along positive and negative extremes of the axes. The light blue wireframes represent average cephalon shape, while the dark blue wireframes represent most extreme shape changes. Different colour dots represent different phylogroups: green dots − Banovina (BAN), black dots − central and south-eastern Europe (CSE), blue dots − Gorski Kotar (GK), purple dots − Lika and Dalmatia (LD), orange dots − Žumberak, Plitvice and Bjelolasica (ŽPB).

Table 6. Procrustes distances between the phylogroups of *A. torrentium* (above diagonal, bold) with *p*-values from permutation tests (10,000 permutation rounds) (below diagonal); Banovina (BAN), central and south-eastern Europe (CSE), Gorski Kotar (GK), Lika and Dalmatia (LD), Žumberak, Plitvice and Bjelolasica (ŽPB).

Phylogroup	BAN	CSE	GK	LD	ŽPB
BAN		**0.0210**	**0.0455**	**0.0307**	**0.0599**
CSE	<0.0001		**0.0452**	**0.0269**	**0.0548**
GK	<0.0001	<0.0001		**0.0337**	**0.0316**
LD	<0.0001	<0.0001	<0.0001		**0.0316**
ŽPB	<0.0001	<0.0001	<0.0001	<0.0001	

Majority of recent studies on the freshwater crayfish morphological variability were based on the statistical analyses of morphometric characteristics using traditional morphometrics. Analyses of large number of morphological characteristics per specimen, in combination with multivariate statistical analyses, enabled the identification of statistically significant differences between populations of the same species, *e.g. A. pallipes* (Ghia *et al.*, 2006; Bertocchi *et al.*, 2008), *A. leptodactylus* (Deniz *et al.*, 2010; Maguire and Dakić, 2011; Benzer et al., 2017), *A. torrentium* (Sint *et al.*, 2006; Maguire *et al.*, 2017), *A. astacus* (Đuretanović *et al.*, 2017) as well as between different crayfish species (Sint *et al.*, 2006, 2007; Larson *et al.*, 2012).

Application of geometric morphometric approach in freshwater crayfish studies was not frequently used (Scalici *et al.*, 2010; Scalici and Bravi, 2012; Helms *et al.*, 2015; Malavé *et al.*, 2018).

Various studies on the crustaceans showed that this group of animals displays great morphological diversity and plasticity (Wills, 1998; García-Dávila *et al.*, 2006; Oda *et al.*, 2007; Stillman *et al.*, 2008; Tanaka, 2009; Zimmermann *et al.*, 2011; Yampolsky *et al.*, 2014). The variations in their phenotype could be a consequence of adaptation to the environment (Zimmermann *et al.*, 2011; Yampolsky *et al.*, 2014) or genetic factors (Atashbar *et al.*, 2016; Hidayani *et al.*, 2018). Likewise, freshwater crayfish exhibit intraspecific morphometric variation that reflects environmental influence (Sint *et al.*, 2005, 2006; Ghia *et al.*, 2006; Haddaway *et al.*,

2012; Perry *et al.*, 2013; Rudolph *et al.*, 2016) or genetic background (Sint *et al.*, 2007; Cataudella *et al.*, 2010; Maguire *et al.*, 2017; Pârvulescu, 2019) or probably combination of both (Baric *et al.*, 2005a, 2005b; Bertocchi *et al. et al.*, 2008; Mathews *et al.*, 2008; Helms *et al.*, 2015; Berger *et al.*, 2017).

Former molecular phylogenetic and phylogeographic research of the stone crayfish based on the mtDNA revealed existence of at least seven (Klobučar *et al.*, 2013) or eight (Pârvulescu *et al.*, 2019) highly divergent monophyletic phylogroups within *A. torrentium*, with the highest genetic diversity recorded in the north-central Dinarids in Croatia. Especially high distances were obtained for phylogroups ZV, GK, ŽPB and LD. Since at that time there was no sufficiently stable diagnostic feature that would reliable distinguish crayfish from different phylogroups, it was suggested that they represent cryptic species (Klobučar *et al.*, 2013). Resolving status of cryptic species is a challenge that requires, beside molecular techniques, morphological approach (Mound *et al.*, 2010; Larson *et al.*, 2012; Singhal *et al.*, 2018). In a small scale preliminary morphometric study of *A. torrentium* populations originating from three phylogroups (ZV, GK and ŽPB) Maguire *et al.* (2017) revealed the characteristics that clearly separate crayfish in a similar way as molecular methods. Current study included larger number of populations per phylogroup, and it covered five out of seven (eight) phylogroups.

Analyses of variance for both sexes displayed significant differences in morphometric characteristics among crayfish belonging to different phylogroups what is in accordance with previous research by Maguire *et al.* (2017). Similarly to results of previous studies (Sint *et al.*, 2005, 2007; Maguire *et al.*, 2017; Mijošek *et al.*, 2017), males differ in more TM characteristics than females. Multivariate discriminant analyses, for both sexes, distinguished characteristics describing claws, head and rostrum (Tabs. 3 and 4) as characteristic that separate populations/phylogroups the best what confirmed results of previous study (Maguire *et al.*, 2017). Percentage of correctly classified female and male stone crayfish per phylogroup was relatively high (from 50 to 100%; Tab. 4) and similar to values obtained in other studies (Sint *et al.*, 2007; Maguire *et al.*, 2017). Misclassification of some of individuals could have been a consequence of paucity of samples caused by limited sampling in certain areas (*e.g.* males in BAN and females in ŽPB) (Larson *et al.*, 2012). Scatterplots of canonical analyses, for both sexes, displayed relatively well separation of individuals from different phylogroups (Fig. 3a and b), what is concordant with previous findings (Maguire *et al.*, 2017). Differentiation between individuals from different phylogroups by CVA and high values of correctly classified males and females per phylogroup indicated clear morphological differentiation of phylogroups and supported the use of traditional morphometrics in phylogroups delimitation.

Geometric morphometrics analyses were applied previously on *A. torrentium*'s sister species *A. pallipes* in order to study allometry during ontogenesis (Scalici and Gibertini, 2009; Scalici *et al.*, 2010) and systematic relations of *A. pallipes* species complex (Scalici and Bravi, 2012). In the present study, significant variation in cephalon shape of *A. torrentium* was observed by GM, and successful intraspecific delimitation, based on morphological variation, was obtained (Fig. 4). Each of the five phylogroups showed a significantly different cephalon shape. The variation was noticeable on the anterior part of the crayfish body, especially rostrum, while the posterior part of the head was less variable in shape, what is consistent with the findings by Bertocchi *et al.* (2008); Larson *et al.* (2012) or Rudolph *et al.* (2016). Results of CVA showed clear separation of phylogroups ŽPB, GK and LD, while there was an overlapping between the phylogroups BAN and CSE. Geometric morphometrics, as a new approach in the research of the stone crayfish morphological features, demonstrate itself as an improvement and complement to TM studies as well as useful in the future population genetics and ecological research of the stone crayfish phylogroups.

Results of both TM and GM canonical discriminant analyses showed congruent topologies: separation among phylogroups, with exception of an overlap of the crayfish from the phylogroups BAN and CSE, which coincides with the results of molecular phylogenetic research of stone crayfish (Klobučar *et al.*, 2013). Further, the results of Mantel test established significant correlation between distances obtained by the two approaches. Correlations between TM and GM were already recorded for different organisms (*e.g.* crustaceans (Malavé *et al.*, 2018; de Melo and Masunari, 2017), honeybees (Tofilski, 2008), cichlids (Parsons *et al.*, 2003), oaks (Viscosi *et al.*, 2009)).

Overall, our results confirmed traditional and geometric morphometrics as useful tools for identification and delimitation of stone crayfish phylogroups and highlighted the urgent need for taxonomic revision of stone crayfish phylogroups status. At the same time, obtained results draw attention to the importance of thorough knowledge about species, including genetics, morphology and ecology, that are necessary for development of proper conservation plans. Application of scientifically based results in conservation measures could help preservation of evolutionary potential and heritage, as well as enable insight into historical biogeography of the species.

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