Geometric vs. traditional morphometric methods for exploring morphological variation of tadpoles at early developmental stages

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Abstract. We conducted a comparative (2D landmark-based geometric and traditional) morphometric analysis on tadpoles at early developmental stages. Two species of brown frog (*Rana dalmatina* and *R. temporaria*) and the common toad (*Bufo bufo*) were involved, all raised in the laboratory from fertilized eggs collected in their natural habitat. Taxonomic identification was confirmed by the DNA barcoding method with the *16S rRNA* sequence as the gene marker. Interested to compare the methodologies for quantification and description of morphological differences among tadpoles of mentioned species, we aimed to: 1) calculate interspecies genetic distances as the most relevant measurement for species differentiation, 2) determine and describe size and shape variation, 3) identify relationships among the analyzed species at the morphological level and 4) assess their classification accuracy. Within the framework of the specified aims, both methodologies produced very similar results, i.e. the smallest divergence was between *R. dalmatina* and *R. temporaria*, while the most discriminative were *B. bufo* and *R. temporaria*. However, we observed subtle shape variation of the distal region of the tail that was detected only by the geometric morphometrics. Our findings support the following. Geometric morphometric method captures more subtle shape differences that were unable to be recovered from linear measurements. It performs slightly better in classification rate. Although it was not quantified, it stands to reason that there is no difference in time investment between the two approaches. Geometric morphometrics provides more information that can be leveraged to answer further questions and it has a clear advantage in visualizing.

Keywords: anurans, DNA barcoding, linear morphometrics, shape, size

Introduction

Most anuran species have a tadpole form that is always bound to an aquatic or very moist habitat (Duellman and Trueb, 1986). Morphological features of tadpoles have been analyzed by applying traditional and geometric morphometric techniques to study their shape evolution in different habitats (e.g., Van Buskirk, 2009, 2017; Baldo et al., 2014; Marques and Nomura, 2015; Sherratt et al., 2018), relationships between morphology and locomotory performance (e.g., burst speed) (e.g., Van Buskirk and Relyea, 1998; Dayton et al., 2005; Arendt, 2010; Johansson, Lederer and Lind, 2010), and the effects of predation (e.g., Ferland-Raymond and Murray, 2008; Johnson et al., 2015), antibiotics and herbicides (e.g., Katzenberger et al., 2014; Peltzer et al., 2017) on tadpole shape. Moreover, as research on tadpoles is expanding, and the tadpoles of some species are visually quite alike, the need for proper taxonomic identification is becoming more urgent (McDiarmid and Altig, 1999).

In this study, we examined three anuran species from the Central Balkans with overlapping breeding seasons and visually similar early tadpole stages. Two of them were brown frogs of the genus *Rana*: *R. dalmatina* Bonaparte, 1840 – the agile frog and *R. temporaria* Linnaeus, 1758 – the common frog (Sillero et al., 2014), while the third species was *Bufo bufo* Linnaeus, 1758 – the common toad. All three species have small, dark-colored tadpoles of similar size and shape (Arnold, 2004; Ambrogio and Mezzadri, 2014). Newly hatched tadpoles of the two *Rana* species are alike in color and shape, which makes taxonomic identification difficult, especially in syntopy (Mc Diarmid and Altig, 1999; Arnold, 2004; Vences et al., 2005). According to Ambrogio and Mezzadri (2014), morphological differences between tadpoles of *R. dalmatina* and *R. temporaria* at early developmental stages are mostly related to tail shape, while the early tadpole stages of *B.*

bufo could be distinguished from those of *Rana sp.* by their eye and vent positions. Additionally, applying the linear morphometric method, Ilić et al. (2016) revealed that relative head length and head width could be suitable discriminative characteristics for tadpoles of *R. dalmatina* and *R. temporaria* and those of *B. bufo* at early developmental stages, while relative tail length could be used to distinguish between tadpoles of the two brown frog species.

In line with future goals given in Ilić et al. (2016), we conducted a comparative (2D landmark-based geometric and traditional) morphometric analysis on tadpoles at early developmental stages. Two species of brown frog and the common toad were involved, all raised in the laboratory from fertilized eggs collected in their natural habitat. For taxonomic identification, we applied the DNA barcoding method with the *16S rRNA* sequence as the gene marker (Ilić et al., 2016). In accordance with previously published papers dealing with morphological variation in tadpoles, we chose a standard set of 2D landmarks (Dayton et al., 2005; Ferland-Raymond and Murray, 2008; Arendt, 2010; Johansson, Lederer and Lind, 2010; Haad, Vera Candioti and Baldo, 2011; Katzenberger et al., 2014; Johnson et al., 2015; Peltzer et al., 2017) and linear measurements (Relyea, 2001; Grosjean, 2005; Alvarez and Nicieza, 2006; Altig, 2007; Arendt, 2010; Hsu et al., 2011; Lima and Pederassi, 2012; Ilić et al., 2016). In order to compare the methodologies for quantification and description of morphological differences among the three species of tadpoles, we aimed to 1) calculate interspecies genetic distances as the most relevant measurement of species differentiation, 2) determine and describe size and shape variation, 3) identify relationships among the analyzed species at the morphological level and 4) assess their classification accuracy.

Materials and methods

Study species

General information on the three species morphologically examined in this study, i.e. *R. dalmatina*, *R. temporaria* and *B. bufo*, have been published elsewhere (see Ilić et al., 2016 and references therein). Characteristics of the spawning sites and tadpole morphology were described by Arnold (2004) and Ambrogio and Mezzadri (2014). *R. dalmatina* tadpoles have an oval and elongated body and thin tail almost twice as long as the body, with a pointed or rounded tip and a well developed fin. The vent opens on the right side, while the spiracle is positioned on the left. Dorsal body color varies from light to dark, while ventrally it is mostly light. In *R. temporaria*, the tadpole body is ovoid, brown or darker, while the tail, approximately twice the body length, is thin and elongated with a blunt tip. The authors also described a flattened depression in the profile line between the eye and the highest point of the tadpole tail fin. Both vent and spiracle openings are oriented in the same directions as in *R. dalmatina*. The *B. bufo* tadpole has an elongated body reaching from 2/3 to 3/5 of the tail length, slightly depressed and dark in color, with eyes located dorsally. The spiracle is left oriented and the vent has a median opening. The tail is longer than the width, with a rounded tip and the dorsal and ventral fins are almost equally developed.

Samples and laboratory procedures

Our samples (egg clutches) were collected in 2015 and 2016 at various localities in the Republic of Serbia (supplementary fig. S_1, supplementary table S_1): four *R. dalmatina* egg clutches (two from Mt. Avala, one from Požega and one from Kragujevac), four *R. temporaria* clutches from Lučani and Grza River (two from each), and four *B. bufo* clutches from Mt. Avala and Grza River (two from each).

The egg masses (12 in total) were stored separately in glass aquaria in the laboratory of the Department of Hydroecology and Water Protection, Institute for Biological Research "Siniša Stanković" (University of Belgrade, Belgrade, Serbia). Tadpoles were hatched in the laboratory, housed in aquaria at densities of approximately five tadpoles per 1.5L and fed with boiled lettuce, rabbit chow and ground fish food (Grosjean, 2005; Johansson, Lederer and Lind, 2010). Aquaria were cleaned on a regular basis once per week. Tadpoles (10 specimens) were randomly

collected from each aquarium at early growth stages (i.e. when they became active swimmers) and preserved in 10% formalin (Dayton et al., 2005; Arendt, 2010). The developmental stages were determined according to Gosner (1960) as 25 – 29.

The overall sample of tadpoles used for morphometric analyses consisted of 120 individuals (40 specimens per species), varying from stage 25 (last hatchling stage) to stage 29 (larva stage). Preserved tadpoles were laid on their right side and the left side was photographed using a Carl Zeiss, Stemi 2000-C binocular magnifier at 6.5x magnification and an AxioCamERc 5s, Zeiss digital camera with ZEN 2011 software.

An additional three tadpoles from each aquarium were subjected to taxonomic genetic identification (according to Ilić et al., 2016) and the obtained *16S rRNA* sequences were deposited under accession numbers MH791090-MH791150 (supplementary table S_1). Interspecies genetic distances were calculated in PAUP v.4.0b (Swofford, 2003). The remaining tadpoles were returned to their habitats of origin.

Geometric morphometric analyses

The tpsDig software (Rohlf, 2015a, b) was used to digitize anatomically or geometrically defined 2D landmarks (fig. 1). Overall 11 landmarks were digitized around the tadpole: (1) head above the eye center, 90° dorsal to the central head region plane, (2) eye center, (3) nostril, (4) snout tip, (5) margin of the upper lip, (6) margin of the lower lip, (7) head below the eye center, 90° ventral to the central head region plane, (8) vent, (9) ventral edge of the tail muscle attaching to the head, (10) dorsal insertion of the tail fin and (11) tip of the tail. To characterize the shape of the tail we collected an additional six landmarks by drawing a line between landmarks 9 and 11, as well as three perpendicular lines at 25%, 50% and 75% of the distance along this line. These additional landmarks were placed at the intersections of the perpendicular lines with the ventral and dorsal margins of the tail. To remove the shape differences due to position of the tail relative to the head, the landmark configurations were straightened by “unbend specimens” operation in tpsUtil (Rohlf, 2015b, 2017; Sherratt et al., 2018). Landmarks 4, 9 and 11 were used to straighten and transform configurations of landmarks.

To remove effects of differences in size, position and orientation, and to obtain shape variables, known as Procrustes coordinates, landmark coordinates were superimposed by generalized Procrustes analysis (GPA) (Rohlf and Slice, 1990; Dryden and Mardia, 1998; Rohlf, 1999). Differences in tadpole size among the studied species were

investigated by analysis of variance (ANOVA) with centroid size (CS), which contains size information, as the dependent variable and species as the independent variable. To explore tadpole shape variation between geographical populations/analyzed species we performed Principal Component Analysis (PCA) of Procrustes coordinates. Shape changes along PC axes were visualized by warped outline graphs (Klingenberg, 2013). Additionally, we examined allometric component of shape variation by conducting multivariate analysis of covariance (MANCOVA), with the first few principal components (PCs) that explained more than 90% of total variance as dependent variables, ln-transformed CS (ln CS) as the covariate and species as the categorical factor. To quantify differences in mean tadpole shape between the analyzed species, Mahalanobis distances (Mds) were calculated from three pairwise comparisons using Discriminant Function Analysis (DFA). To estimate the statistical significance of the observed Mds, a permutation test with 10,000 permutation runs was used. Finally, classification accuracy of the analyzed species was assessed by applying leave-one-out cross-validated DFA (Lachenbruch, 1967; Viscosi and Cardini, 2011; Jojić et al., 2014). The analyses of variance and covariance (ANOVA and MANCOVA) were done in Statistica (StatSoft, 2004), whereas all other analyses were performed using MorphoJ (Klingenberg, 2011).

Traditional morphometric analyses

From the same images used for geometric morphometrics, ten linear measurements (fig. 1) were recorded with the TMorphGen6 program, Integrated Morphometrics Program (IMP) series (Sheets, 2000). Linear measurements were as follows: HH – head height, HL – head length, E – eye diameter, TH – tail height, TL – tail length, CC – central tail muscle, VT – distance between vent and tip of the tail, DIT – distance between dorsal insertion of the tail fin and tip of the tail, DIS – distance between dorsal insertion of the tail fin and snout tip, VS – distance between vent and snout tip. Grey landmarks in Figure 1 were used only for calculation of linear measurements HH, E, TH and CC. To adjust linear data for the isometric effects of size, we performed the Mosimann's log-shape ratios approach (Mosimann, 1970; Mosimann and James, 1979; Klingenberg, 2016), i.e. each of the measurements was divided by a standard size variable that quantifies the overall size of the object (represented by the geometric mean of the measurements – GM) and ln-transformed. This approach is considered as analogous to the size correction in the Procrustes superimposition method for landmark data (Sherratt et al., 2017). However, besides the geometric mean (GM) of the measurements, examples of standard size variables are any one of the original measurements, the arithmetic mean of the

measurements or any linear combination of log-transformed measurements for which the coefficients sum up to 1.0 (Klingenberg, 2016).

Differences in tadpole size among the studied species were investigated by two separate ANOVAs with the overall size (represented by GM and total tadpole length – TOTL in first and second ANOVA, respectively) as the dependent variable and species as the independent variable. In addition, each of the GM-size-adjusted variables was compared among the species by one-way ANOVA followed by Bonferroni correction. To examine tadpole shape variation between geographical populations/analyzed species, we performed PCA of linear data size-adjusted by GM and ln-transformed. To explore allometric component of shape variation, we conducted two separate MANCOVAs with the first few PCs that explained more than 90% of total variance as dependent variables and species as the categorical factor. Ln GM and ln TOTL was used as the covariate in first and second MANCOVA, respectively. For linear data set size-adjusted by GM and ln-transformed we quantified differences between tadpoles of the analyzed species by calculating squared Mahalanobis distances (Mds) from three pairwise comparisons and classification accuracy was assessed by leave-one-out cross-validated DFA (Lachenbruch, 1967; Viscosi and Cardini, 2011; Jojić et al., 2014). Analyses of variance and covariance (ANOVA and MANCOVA), PCA and calculation of Mds were done in Statistica (StatSoft, 2004), whereas DFA was performed in SPSS 21.0 (IBM SPSS Statistics for Windows, 2013. Armonk, NY: IBM Corp).

Results

The greatest mean interspecies genetic distance was between *B. bufo* and *R. temporaria* sequences (mean ± SD = 0.1982 ± 0.0072), followed by *B. bufo* and *R. dalmatina* (0.1887 ± 0.0036). As expected, the lowest distance was between *R. dalmatina* and *R. temporaria* (0.0464 ± 0.0036).

Geometric morphometrics

Analysis of variance (ANOVA) showed no significant differences in body size among three analyzed species ($F_{2,117} = 0.84$, $P = 0.4324$). The mean values, standard deviations and standard errors of centroid size (CS) for each species are graphically presented in Figure 2A.

As shown on the Principal Component Analysis (PCA) graph (fig. 3A), the PC1 axis (accounting for 66.1% of the shape variation) clearly segregated *B. bufo* from two *Rana* species. In comparison to those of *R. dalmatina* and *R. temporaria*, *B. bufo* tadpoles were generally more robust and characterized by considerably larger bodies with dorsal fins placed posteriorly, while their tails were shorter with wider posterior parts of fins and larger tip depths. The head and tail had approximately the same length in *B. bufo* tadpoles, while in both *Rana* species the tail was almost twice as long as the head. The PC2 axis (12.5% of the variation) separated *R. temporaria* (placed in the positive part of the axis) from *R. dalmatina* (with negative scores on the axis). In comparison to *R. dalmatina*, *R. temporaria* tadpoles had longer tails with more pointed tips and smaller heads with lower dorsal insertions of the tail fins. In addition, PCA indicated that geographical populations of the same species clustered together.

Multivariate analysis of covariance (MANCOVA), conducted on the first five principal components (PCs) that explained 91.5% of total variance, revealed statistically significant shape differences between tadpoles belonging to the three analyzed species ($\lambda_{Wilks} = 0.7314$, $F_{10,220} = 3.72$, $P = 0.0001$). Additionally, significant contribution of size to the shape variation pointed to allometry ($\lambda_{Wilks} = 0.6878$, $F_{5,110} = 9.99$, $P = 0.0000$), while significant interaction between size and species indicated that different species have different allometric slopes ($\lambda_{Wilks} = 0.7771$, $F_{10,220} = 2.96$, $P = 0.0017$).

Interspecific differences in tadpole shape were quantified by Mahalanobis distances (Mds). The smallest divergence in larval shape was found between *R. dalmatina* and *R. temporaria* (Md = 10.5808, $P < 0.0001$), followed by *B. bufo* and *R. dalmatina* (Md = 14.1408, $P < 0.0001$), while the largest was between *B. bufo* and *R. temporaria* (Md = 15.8947, $P < 0.0001$). Based on cross-validated DFA, 100% of the specimens were reclassified correctly in their species of origin.

Traditional morphometrics

Analysis of variance (ANOVA) revealed no significant differences in overall tadpole size, i.e. analyzed species differ neither in geometric mean (GM) ($F_{2,117} = 0.03$, $P = 0.9678$) nor in total tadpole length (TOTL) ($F_{2,117} = 1.70$, $P = 0.1865$). For each species, the mean values, standard deviations and standard errors of GM and TOTL are graphically presented in Figure 2B and Figure 2C, respectively. Results of one-way ANOVA tests for each of the ten GM-size-adjusted variables are summarized in Table 1. All variables showed statistically significant differences among the analyzed species ($P < 0.001$).

As presented on the Principal Component Analysis (PCA) graph (fig. 3B), the PC1 axis (accounting for 48.5% of variance) separated the two *Rana* species from *B. bufo*, while tadpoles of the two brown frog species were distinguished along the PC2 axis (accounting for 20.6% of variance). PCA analysis also revealed that along PC1 axis the most discriminative characters among species were those related to tail (TL, VT, DIT) and head (DIS, HL) lengths, while eye diameter (E) and measurement associated with the tail muscle width (CC) contributed to species separation along PC2 axis (Table 2). Mean values and standard deviations of ten linear variables size-adjusted by geometric mean (GM) are given in Table 3. In comparison to two *Rana* species,

B. bufo tadpoles were characterized by smaller average values for tail, but greater average values for head lengths. Tadpoles of *R. dalmatina* (positive section of the PC2 axis) showed higher mean eye diameter than those of *R. temporaria* (negative section of the PC2 axis), whereas *R. temporaria* tadpoles had wider central tail muscle (CC). Finally, PCA disclosed that geographical populations of the same species grouped together.

Multivariate analyses of covariance (MANCOVAs) were conducted on the first five principal components (PCs) that explained 94.2% of total variance. Both MANCOVAs showed quite similar results: statistically significant shape differences between tadpoles belonging to the three analyzed species (MANCOVA with ln GM as the covariate: $\lambda_{Wilks} = 0.6374$, $F_{10,220} = 5.56$, $P = 0.0000$; MANCOVA with ln TOTL as the covariate: $\lambda_{Wilks} = 0.2625$, $F_{10,220} = 20.94$, $P = 0.0000$), significant effects of size indicating allometry (MANCOVA with ln GM as the covariate: $\lambda_{Wilks} = 0.8585$, $F_{5,110} = 3.63$, $P = 0.0045$; MANCOVA with ln TOTL as the covariate: $\lambda_{Wilks} = 0.8581$, $F_{5,110} = 3.64$, $P = 0.0044$) and significant interaction between size and species, i.e. heterogeneity of allometric slopes among species (MANCOVA with ln GM as the covariate: $\lambda_{Wilks} = 0.8059$, $F_{10,220} = 2.51$, $P = 0.0072$; MANCOVA with ln TOTL as the covariate: $\lambda_{Wilks} = 0.7870$, $F_{10,220} = 2.80$, $P = 0.0028$).

Interspecific differences in tadpoles were quantified by Squared Mahalanobis distances (Mds). As expected, *R. dalmatina* and *R. temporaria* distance had the lowest value (Md = 24.2712, $F_{9,109} = 50.25$, $P < 0.001$), followed by *B. bufo* and *R. dalmatina* (Md = 25.1628, $F_{9,109} = 52.09$, $P < 0.001$), while the largest was between *B. bufo* and *R. temporaria* (Md = 51.3068, $F_{9,109} = 106.22$, $P < 0.001$). Cross-validated DFA showed that 99.2% of the specimens were reclassified correctly in their species of origin.

Discussion

In this paper we compared 2D landmark-based geometric and traditional morphometric methodologies used for discrimination of laboratory-reared tadpoles of *R. dalmatina*, *R. temporaria* and *B. bufo* at early developmental stages. Their taxonomic identification was confirmed using the DNA barcoding method. Within the frame of the specified aims, we applied the standard procedures used in studies dealing with morphological variation. Although both methodologies produced very similar results, we observed some differences between them primarily related to description of tail shape. More precisely, subtle differences of the distal region of the tail could be detected only by the geometric morphometric method.

Interspecies genetic distances observed herein are concordant with previous outcomes of phylogenetic analysis of these three species (Ilić et al., 2016). According to interspecies morphological and genetic distances, geometric as well as traditional morphometrics disclosed that tadpole shape variation coincides with their genetic differentiation. The smallest divergence was between *R. dalmatina* and *R. temporaria*, while the most discriminative were *B. bufo* and *R. temporaria*. Considering quantification and description of size and shape variation, the geometric and traditional morphometric approaches gave similar results. Namely, whether centroid size (CS), geometric mean (GM) or total tadpole length (TOTL) was used as overall size, no statistically significant size differences were observed between the analyzed species. However, multivariate analyses revealed statistically significant shape differences and allometry as a possible driving factor in the observed shape variation. Principal Component Analysis (PCA) scatterplots (Figure 3) pointed to populations clustering within the analyzed species and to the same pattern of species discrimination along the PC1 and PC2 axes, i.e. the PC1 axis separated *Rana* species from *B. bufo*,

while *R. temporaria* was distinguished from *R. dalmatina* along the PC2 axis. Furthermore, both morphometric approaches described shape of the analyzed tadpoles in a similar way (fig. 3A; Tables 1, 2 and 3). In contrast to *B. bufo*, the *Rana* species tadpoles were generally slimmer with smaller bodies and tails approximately twice as long as the body length. When the two *Rana* species were compared, *R. dalmatina* tadpoles had smaller tails and larger bodies. However, subtle differences in the distal region of the tail could be detected only by the geometric morphometric method. As evident from Figure 3A, *Rana* species tadpoles had narrower posterior parts of the fins and smaller tip depths than those of *B. bufo*, while in comparison to *R. temporaria*, the tails of *R. dalmatina* tadpoles had more rounded tips. Since the linear method only measures maximal tail length and height (Relyea, 2001; Grosjean, 2005; Alvarez and Nicieza, 2006; Altig, 2007; Arendt, 2010; Hsu et al., 2011; Lima and Pederassi, 2012; Ilić et al., 2016), the geometric morphometric approach could capture more subtle variation in tail shape, particularly that related to the distal region.

The discrimination power of DF analysis provided 100% and 99.2% accuracy in geometric and traditional morphometrics, respectively. Fairly high discrimination power (96% of tadpoles correctly classified) was also obtained by Escoriza and Hassine (2014) in their outline-based geometric morphometric study of two *Pelobates* species. However, using a digital caliper for linear measures of tadpoles, Arendt (2010) compared linear and geometric morphometric methods to distinguish between five species of spadefoot toads (Scaphiopodidae). Geometric morphometrics was superior, correctly assigning 98% of individuals while the traditional method correctly assigned just 70% of the specimens. Almost equal values of classification accuracy obtained here with the applied methodologies could be due to procedural issues during traditional morphometrics, i.e. our linear measurements were taken from images instead of by caliper.

In a review of the use of geometric morphometrics in herpetology, Kaliontzopoulou (2011) remarked that many of authors who have compared geometric to traditional morphometric methods concluded that geometric morphometrics was frequently more powerful than linear morphometrics for detecting and describing organismal shape variation (Valenzuela et al., 2004; Bonnan, Farlow and Masters, 2008; Kaliontzopoulou, Carretero and Llorente, 2008; Arendt, 2010). However, our results presented herein point to general similarity of the applied methodological approaches. The main difference between geometric and traditional morphometrics observed in our study is related to description and detection of shape variation in the distal region of the tail. This dissimilarity is probably due to the following fact. The standard set of linear measurements lacks those related to the distal part of the tail, while the standard set of 2D landmarks includes those positioned on the distal part of the tail. Therefore, variability in this part of the tail could be detected only by geometric morphometrics. Similarly, analyzing the relationship between tadpole morphology and swimming speed in five species of spadefoot toad tadpoles (Scaphiopodidae), Arendt (2010) found that faster swimmers also had deeper tails especially in posterior part and concluded that this pattern would have been missed in linear morphometrics which usually only measures maximal tail depth.

Differences in methods (including the choice of linear measurements and landmarks) can lead to discrepancies in the results. Therefore, direct comparison among studies that use traditional and geometric morphometric methods (including those dealing with tadpole morphological variation) may be reliable and likely produce similar results when the scheme for linear measurements corresponds to the scheme for configuration of landmarks, i.e. when using an adequate number and distribution of linear measurements. Accordingly, Jojić, Blagojević and Vujošević (2012) disclosed that geometric and traditional morphometric methods for testing two-

module organization of the mandible produce very similar results when applied to the patterns of covariation/correlation in morphological data of the yellow-necked field mouse. Likewise, Jojić et al. (2014) successfully discriminated *Apodemus flavicollis* and *A. sylvaticus* from Serbia using geometric and traditional morphometric methods on a data set for ventral crania of specimens previously genotyped by the Inter Simple Sequence Repeat-PCR (ISSR-PCR). They showed that the discrimination power of the applied approaches was more or less similar. Finally, using both linear and geometric morphometric techniques, Larson (2002) demonstrated that chondrocranial growth is not isometric and that regionally distinct patterns of shape change are present in larval *R. sylvatica*. He concluded that the results of linear and geometric morphometric analyses were largely congruent.

Considering the use of traditional and geometric morphometrics in studies of morphological variation in tadpoles, our findings support the following. Geometric morphometric method captures more subtle shape differences that were unable to be recovered from linear measurements and can serve as a complement to other measures commonly used in traditional morphometrics (Arendt, 2010; Escoriza and Hassine, 2014). Furthermore, it performs slightly better in classification rate and has a clear advantage in visualizing. Besides, as tadpoles of *R. dalmatina*, *R. temporaria* and *B. bufo* at early developmental stages are rather small (average values for total tadpole length - TOTL range from about 14 to 15 mm; fig. 2C), they are difficult to handle and taking linear measurements by caliper could be imprecise. Therefore, the use of their images is a better choice. Although it was not quantified, it stands to reason that there is no difference in time investment between taking linear measurements from images and digitizing 2D landmarks. Finally, geometric morphometrics provides more information that can be leveraged to answer further questions. For example, studying shape of different larval stages belonging to the

same species could help in resolving taxonomic and phylogenetic problems where adult characters alone have been inadequate (Grosjean, 2005). Patterns of tadpoles shape changes could also be quantified, visualized and compared in studies dealing with static, ontogenetic or evolutionary allometry, adaptations and phenotypic plasticity, as well as in the light of systematics, taxonomy and phylogeny of anurans (Kaliontzopoulou, 2011). All of these facts emphasize the advantages of geometric over traditional morphometrics, at least for exploring morphological variation of tadpoles at early developmental stages.

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Table 1. Analysis of variance (ANOVA) for each of the ten linear measurements size-adjusted by the geometric mean (GM) with species as the categorical factor. HH – head height, HL – head length, E – eye diameter, TH – tail height, TL – tail length, CC – central tail muscle, VT – distance between vent and tip of the tail, DIT – distance between dorsal insertion of the tail fin and tip of the tail, DIS – distance between dorsal insertion of the tail fin and snout tip, VS – distance between vent and snout tip. Statistical significance (*P*) after Bonferroni correction.

	df1, df2	*F*	*P*
HH	2, 117	113.92	< 0.0001
HL	2, 117	46.76	< 0.0001
E	2, 117	20.86	< 0.0001
TH	2, 117	10.36	< 0.001
TL	2, 117	190.60	< 0.0001
CC	2, 117	32.06	< 0.0001
VT	2, 117	178.14	< 0.0001
DIT	2, 117	196.37	< 0.0001
DIS	2, 117	175.57	< 0.0001
VS	2, 117	21.14	< 0.0001

Table 2. The factor loadings obtained from Principal Component Analysis (PCA) carried out on the traditional morphometric data size-adjusted by the geometric mean (GM) and ln-transformed (see Fig. 3B). Factor loadings above 0.8 are in bold.

	PC1	PC2
HH	-0.6204	0.4935
HL	**-0.8202**	-0.1359
E	0.2077	**0.8269**
TH	0.2921	-0.4330
TL	**0.9315**	-0.2005
CC	-0.1061	**-0.8412**
VT	**0.9188**	-0.2212
DIT	**0.9178**	0.0915
DIS	**-0.8236**	-0.2748
VS	-0.6464	-0.2165

Table 3. Means and standard deviations (SD) for ten linear variables size-adjusted by the geometric mean (GM).

	R. dalmatina		*R. temporaria*		*B. bufo*	
	Mean	SD	Mean	SD	Mean	SD
HH	0.825	0.068	0.688	0.036	0.878	0.065
HL	1.498	0.049	1.475	0.035	1.589	0.076
E	0.124	0.012	0.107	0.010	0.112	0.015
TH	0.892	0.042	0.909	0.046	0.863	0.048
TL	2.423	0.124	2.561	0.064	2.162	0.081
CC	0.275	0.015	0.302	0.012	0.290	0.019
VT	2.293	0.111	2.414	0.078	2.028	0.088
DIT	3.091	0.187	2.980	0.115	2.466	0.140
DIS	0.931	0.101	1.085	0.092	1.356	0.114
VS	1.696	0.059	1.704	0.035	1.781	0.087

Figure 1. 2D landmarks and linear measurements collected on the tadpoles.

Figure 2. Plot of centroid size - CS (A), geometric mean - GM (B) and total tadpole length - TOTL (C) means, standard deviations and standard errors for the analyzed species.

Figure 3. Scatterplot of the first two Principal Components (PC1 vs. PC2) from the Procrustes coordinates. Shape changes are presented as warped outline graphs along the PC1 and PC2 axes (A). Scatterplot of the first two Principal Components (PC1 vs. PC2) from linear measurements size-adjusted by the geometric mean (GM) and ln-transformed (B); see Table 2 for factor loadings obtained from this PCA. Specimens of the same species are joined by outline polygons.