IDENTIFICATION OF SYNTOPIC ANURAN SPECIES IN EARLY TADPOLE STAGES: CORRESPONDENCE BETWEEN MORPHOMETRIC AND GENETIC DATA

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Abstract. Many European frogs and toads are relatively secretive species and except during breeding season, adults can rarely be seen during time-restricted fieldwork. In contrast, their tadpoles are easy to record and could be very useful in a brief biodiversity assessment. It is important to perform quick and accurate taxonomic identification of tadpoles, yet genetic methods are costly and cannot be routinely applied. We tested suitability of morphometric analysis for taxonomical distinction among tadpoles of early breeding local anuran species. Tadpole samples were collected simultaneously at three different locations in Republic of Serbia (South-eastern Europe) in habitats known to be breeding sites shared by brown frogs and the common toad. DNA barcoding verified *Rana dalmatina*, *R. temporaria* and *Bufo bufo* species, each collected in different location. The results of linear morphometric analyses suggested that relative head length and head width could be good discriminative characteristics for tadpoles of these two *Rana* species and those of *B. bufo*. To distinguish between tadpoles of two analyzed brown frog species, relative tail length could be used. For further development of the identification procedures for tadpoles of particular species, it is essential to involve geometric morphometrics and to analyze different larval developmental stages.
Keywords: *brown frogs, common toad, early breeders, taxonomic identification*

Introduction

Analyses of tadpole morphology have been shown as very applicable in anuran taxonomy and phylogeny (Duelmann and Trueb, 1994; Sidorovska et al., 2002; Grosjean, 2005; Vejarano et al., 2006), but nowadays they become interesting also for conservation studies in a broadest sence (Buskirk, 2009; Severtsova et al., 2012; Pujol-Buxo, 2013; Schulze et al., 2015). Some anuran genera include morphologically similar and partly syntopic taxa and their species can be recognized mainly on the basis of genetic differences and differences in advertising calls (Larson and Chippindale, 1993). Moreover, many European frogs and toads are secretive crepuscular or nocturnal species so adults, except during the breeding season, can rarely be seen during short visits to the place (Arnold and Ovenden, 2002). In contrast to adult individuals, their tadpoles are easily detectable through the whole aquatic life stage (McDiarmid, 1994). In such cases, confident taxonomic identification of tadpoles is sometimes the only way

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to do quick and complete assessment of anuran fauna in the area of interest (Gascon, 1991). Although topics relating to ecological studies and environmental impact assessments are demanding, genetic methods still cannot be routinely applied due to restricted funds, which is a common problem in such studies.

Morphometric analysis of tadpoles has been widely used in interspecific comparisons, but often based on the ratios of total length or snout-vent length, where intra-specific and intra-populational variability should be taken into consideration (Sidorovska et al., 2002; Grosjean, 2005; Arendt, 2010). Regarding the tadpole's morphology, there are two phases with remarkable changes: before stage 25 and after stage 42 (McDiarmid and Altig, 1999). Many authors described various tadpole larval phases in their work (details in Lima and Pederassi, 2012) but most of these studies focus on the stages between 37 and 39 (Lima and Pederassi, 2012).

The aim of this paper was to evaluate the use of tadpole morphometric analysis for taxonomic distinction among locally-occurring syntopic, early-breeding anuran species. Unlike other studies, our work was focused on early development stages (hatchlings, stages 23 to 25, according to Gosner, 1960), as at this phase distinction among those species is sometimes difficult in the field (Arnold and Ovenden, 2002). The development of procedures for confident taxonomic identification of anuran tadpoles, including hatchlings, could help in more effective faunistic and ecological surveys of amphibians. This is particularly important in areas where the narrow zones of sympatry and occurrence of syntopy among various anuran species are recorded, such as in Republic of Serbia in South-eastern Europe (Dufresnes et al., 2013; Vukov et al., 2013).

Material and Methods

The brown frogs of genus *Rana* (family Ranidae) are among the earliest-breeding European anurans; the adults are mostly terrestrial, with an aquatic larval stage. Some European brown frog species spawn in fast highland streams (e.g. *R. graeca* in the Balkans; Arnold and Ovenden, 2002) but most breed in various types of stagnant or moderately fast-running waters, occurring in lowland habitats and at altitudes up to 2745 m a.s.l. in the Alps (Veith et al., 2003). It has been noted that Eurasian brown frogs are sometimes difficult to classify (Che et al., 2007) which is an issue particularly in the areas of syntopy.

Three species of brown frog, namely, *Rana dalmatina*, *R. temporaria* and *R. graeca* are common in South-east Europe (Sillero et al., 2014) with *R. dalmatina* and *R. temporaria* being widely distributed throughout Europe (Gasc et al., 1997; Arnold and Ovenden, 2002; Sillero et al., 2014). As noted in Hartel (2005), syntopic habitats of these two species are rare and interspecific competition may contribute to their niche separation (Riis, 1988). A study along the Târnava Valleys in Romania showed that the domination of *R. dalmatina* over *R. temporaria* was a common phenomenon in the lower to middle parts of the Valley, while in the upper section (>600 m elevation) *R. temporaria* began to dominate (Hartel, 2005). On the contrary, the long-term studies in Western Europe (Gollmann et al., 2002) showed an inverse relationship i.e. the domination of *R. temporaria* upon *R. dalmatina* (Hettyey and Pearman, 2003; Hartel, 2005). *R. graeca* is a brown frog species, endemic in the Balkan Peninsula, where it inhabits river gorges and canyons at altitudes from 300 m to 1000 m (Asimakopoulos, 1997).

We collected samples of tadpoles in early spring 2013 in three locations in Serbia, where the occurrence of brown frog species was common knowledge (Crnobrnja, 1982;

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Arnold and Ovenden, 2002; Tomašević et al., 2008). In Serbia, the agile frog (*R. dalmatina*) has a widespread distribution (Vukov et al., 2013). In contrast, the grass frog (*R. temporaria*) has a rather scattered distribution and for this reason is considered to be a species of conservation concern (Crnobrnja-Isailović and Paunović, 2015; Vukov et al., 2015). Both agile and grass frogs start breeding activities shortly after the snow has melted and their mating season ends quickly (Hartel, 2005; Iosob and Prisecaru, 2014; Crnobrnja-Isailović et al., 2015). *R. graeca* mostly breeds in cool and fast-running watercourses in hilly and mountainous areas (Arnold and Ovenden, 2002) and its occurrence in certain locations in Serbia overlaps with either *R. dalmatina* or *R. temporaria* (Vukov et al., 2013).

The common toad (*Bufo bufo*) is the only other anuran species in the area that breeds almost as early in the spring as brown frogs (Arnold and Ovenden, 2002). Both the eggs masses and the tadpoles of these two genera, *Bufo* and *Rana*, are easily distinguished (Arnold and Ovenden, 2002). However, it could not work on very early developmental stage: shortly after hatching, the tadpoles of brown frogs and the common toad could have similar appearance and cannot be taxonomicaly identified *in situ* by simple visual inspection.

Description of sampling locations

Geographic position of sampling locations is presented in *Table 1* and *Fig. 1*.

Figure 1. *Geographic position of sampling locations.*

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Table 1. *Geografic coordinates of the sampling locations.*

Location	Longitude	Latitude	Altitude (*m*)
Zuce reservoir	44º 40' 55.9"	20º 33' 7.4"	240
Bigar Hill	44º 13' 26"	21º 52' 20"	720
Trešnjica River gorge	44º 07' 18.46"	19º 29' 57.3"	200

Each sampling location is distinctive regarding habitat characteristics and anthropogenic pressure. Lake Zuce, an artificial water reservoir, is situated in an agricultural area at the foot of Mountain Avala near the capital of Republic of Serbia, Belgrade, so the area is under considerable anthropogenic pressure. Geologically, Mt. Avala consists of limestone, marl, sandstone and serpentine rock. It is a conical hill, mostly covered by forest vegetation, both native and planted, trees including durmast oak (*Quercus petraea*), Turkish oak (*Quercus cerris*), hornbeam (*Carpinus betulus*), beech (*Fagus sylvatica*), linden (*Tilia europaea*), black pine (*Pinus nigra*) and black locust (*Robinia pseudoacacia*). Meadow vegetation is also present, but less extensive. The lake itself is surrounded by remnants of the deciduous forest (Tomašević et al., 2008). The only brown frog species recorded there is *R. dalmatina* (Crnobrnja-Isailović et al., 2012).

In comparison with the Zuce Reservoir, the other two sampling sites are under lower anthropogenic influence. The hilly-mountainous stream of Bigar is located on Bigar hill, part of the Homolje mountain range in eastern Serbia. The hill is composed of limestone and its vegetation consists of beech forest communties (*Acero Carpinetum betuli* [maple and hornbean]), *Fagetum montanum* (mountain beech) and *Fagetum montanum subas, Corydalo Fagetum* (community of beech with Holewort and Spring fumewort) and *Acero Fagetum* (beech with maple). In early spring, these communities are dominated by annual species, mostly ground flora, such as *Corydalis solida, Corydalis cava, Dentaria bulbifera* etc. Also, the area is covered in grassland, in the form of mowed meadows. The Bigar stream is one of the biggest permanent streams in this area. The stream bed is up to 1m in width and 30cm deep on average, and it joins with the Valja Saka stream to form the Jagnjilo River, which continues to flow to the north. Like all watercourses in this part of Serbia, Bigar stream belongs to the Danube Basin (Paunović et al., 2014). Two brown frog species occur in the area – *R. dalmatina* and *R. temporaria* (Crnobrnja, 1982).

Trešnjica River (2-3m in width and up to 50cm deep) is located in western Serbia. This clean mountain river emerges below Povlen Mountain in western Serbia, and after 23 km it flows into the Drina River, also belonging to the Danube catchment. Immediately before joining the Drina, Trešnjica flows through a several-kilometre long limestone gorge, in places characteristic of a canyon valley. The vegetation mainly comprises Oriental hornbeam (*Carpinus orientalis*), Turkish oak (*Quercus cerris*), Italian oak (*Quercus frainetto*), black pine (*Pinus nigra*), prickly juniper (*Juniperus oxycedrus*), and several other thermophilous species. Trešnjica River gorge is one of Serbia's nature reserves (Amidzić et al., 2007). The only known brown frog species spawning in this river is *R. graeca* (Arnold and Ovenden, 2002).

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Sampling procedure

On every locality, ten individuals were gathered from the same aggregation of tadpoles by a standard deep or hand net, and they were preserved in 70% ethanol. In laboratory, tissue samples for genetic analyses (e.g. tip of the tail) were taken after measurement procedure and deposited in 95% ethanol. The collected tadpoles were in early development stages – hatchlings (stages 23 to 25, according to Gosner, 1960). Their body colour in all samples was black, while only specimens from Bigar Hill had visible external gills. Samples were deposited at the Department of Hydro-ecology and Water Protection, Institute for Biological Research "Siniša Stanković", University of Belgrade. Collection permit was issued by Ministry of Energetics, Development and Nature Protection of Republic of Serbia, No. 353-01-54/2013-08.

DNA extraction, amplification and sequencing

We have randomly chosen three of ten tadpoles from each locality from the same dense aggregation of tadpoles for genetic identification. Total DNA was extracted from 5 mm of tadpole's tail muscle, using the *AccuPrep* Genomic DNA Extraction kit, according to the manufacturer's instruction (Bioneer Corporation, Daejeon, R. Korea). The tissue was incubated in a tissue lysis buffer at 50°C, for one hour. After precipitation and washing, DNA was eluted using a 75microL elution buffer. Depending on the species, an (approximately) 380 bp fragment of mitochondrial *16S rRNA* gene was amplified by PCR with termalprofile and primers (16Sar: 5'-CG CCTGTTTATCAAAAACAT-3' 16Sbr: 5'-CCGG TCTGAACTCAGATCACGT-3') described in Veith et al. (2003). Special care was taken to ensure sterile conditions, and for each PCR run negative control (with water instead of a template) was used as a contamination check. Amplicons were sequenced in both directions using a BigDye® Terminator v3.1 Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA) and sequences were base called and assembled with ABI software: Sequencing Analysis 5.1 and SeqScape software, v 2.5. The obtained sequences were deposited in GenBank database (http://www.ncbi.nlm.nih.gov/genbank) under accession numbers KR136355 - KR136364 (Appendix 1).

Additional sequences for analysis were downloaded from GenBank. All sequences were aligned by ClustalW (Thompson et al., 1994) and visually inspected in Bioedit 7.2.5 (Hall, 1999). A best-fit substitution model in aligned sequences was examined by JModelTest v.2.1.4. (Darriba et al., 2012) while phylogenetic analysis was conducted using MEGA 6.0 (Molecular Evolutionary Genetics Analysis software; Tamura et al., 2013). Tadpole species were identified using a DNA barcoding approach, based on sequenced *16S rRNA* gene part.

The Maximum Likelihood (ML) method, integrated in MEGA 6.0 software, was used to build a phylogenetic tree. Bootstrap analysis was done to determinate the strength of support for a clade on the phylogenetic tree.

The *Bombina variegata* species' sequence was used to root the tree.

Morphometric analysis

The tadpoles were placed in a Petri dish with constantly refreshed water, in order to avoid desiccation. The subjects' dorsal, lateral and ventral sides were photographed using a binocular magnifier Carl Zeiss, Stemi 2000-C with magnification 6.5 and a

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digital camera AxioCamERc 5s, Zeiss. ZEN 2011 software and ImageJ (Abramoff, 2004) were used for all measurements.

Morphometric characteristics used in subsequent analyses were chosen in accordance with available literature (Van Buskirk and Relyea, 1998; Sidorovska et al., 2002; Vences et al., 2002; Dey and Gupita, 2002; Grosjean, 2005; Vejarano et al., 2006; Altig, 2007; Arendt, 2010; Di Cerbo and Biancardi, 2010; Severtsova et al., 2012; Johansson and Richter-Boix, 2013). These were: ed – eye distance, hh – head height, e, – eye diameter, tl – tail length, cc – central tail muscle, th – tail height, m – mouth length, hwv – head width, hlv – head length and bl – body length. Measurements were standardized by body length (bl) to obtain standardized values for further analyses (ED – eye distance, HH – head height, E – eye diameter, TL – tail length, CC – central tail muscle, TH – tail height, M – mouth length, HWV – head width, HLV – head length).

The normality test (Shapiro-Wilk's test) was used to determine if the data set had normal distribution. Descriptive statistics were used to calculate various measurements, such as mean, standard deviation, minimum and maximum, range, median etc. of the obtained data. Levene's test of equality of variances between variables was used to assess homogenity of data (Levene, 1960). Test of equality of covariances (Box's test) (Box, 1949) was used to assess homogenity of covariance matrices. One-way ANOVA with contrasts was used to analyze the significance of differences between group means for tested variables. The linear regression was applied for modeling the relationships between tested variables. Pearson product-moment correlations were used to check linear correlation between analyzed variables. Uncorrelated variables were excluded from further multivariate analysis (Canonical Discriminant Analysis; CDA). The CDA was used to determine which variables discriminate analyzed data of these naturally co-occurring groups, and to visualize its relationships (Quinn and Keough, 2002; Young and Young, 1998; Simonović, 2004; Ivanović and Kalezić, 2009; Hair et al., 2010).

Performed statistical analysis were done by using the software package Statistica 7.0 (StatSoft, 2004).

Results

Phylogenetic analysis

The final dataset for phylogenetic analyses included 27 sequences from GenBank and 10 sequences obtained during our analysis (*Appendix 1*). As there is a lack of *R. graeca* sequences in GenBank, especially in the case of *16S rRNA* gene, we sequenced the tissue sample (a fingertip) collected earlier from one *R. graeca* adult from Serbia (Access no. KR136364). After careful examination, obtained sequences of *16S rRNA* gene, 421 - 537 bp long, were cut to 348 bp reliable sequence and used in phylogenetic analysis. The performed model test determined Tamura Nei model (1993) with invariant sites (TN93+I) as the most suitable. The maximum likelihood (ML) method produced a tree (*Fig. 2*) with good support for the analyzed species.

Two main clades were distinguished on obtained phylogenetic tree corresponding to analyzed *Rana* and *Bufo* gene sequences. The *Rana* clade included two subclades - one containing *R. temporaria* and the other with *R. dalmatina* and *R. graeca*. Within *R. dalmatina,* samples from Serbia formed a separate cluster, with good support from other analyzed conspecific sequences from Moldova, Spain, Germany and France. Within *R. temporaria* subclade, two main lineages were observed. Samples from Serbia were clustered with those from Spain, Czech Republic, Sweden, Russia and Ukraine. The

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other lineage contained conspecific sequences, mostly from the Western Europe. The *Bufo bufo* clade was well defined in the obtained tree (consider that *B. bufo* PT sequence information taken from the GenBank is in fact *Bufo spinosus* PT, due to taxonomic revision of *Bufo bufo* species group after the paper by Recuero et al., 2012). Besides samples from Greece, samples from Serbia also formed a separate cluster. We obtained uncosistent results regarding sample from Trešnjica river: DNA analysis showed that those tadpoles belong to *B. bufo* rather than to *R. graeca* what was expected based on geographical position and type of the breeding site.

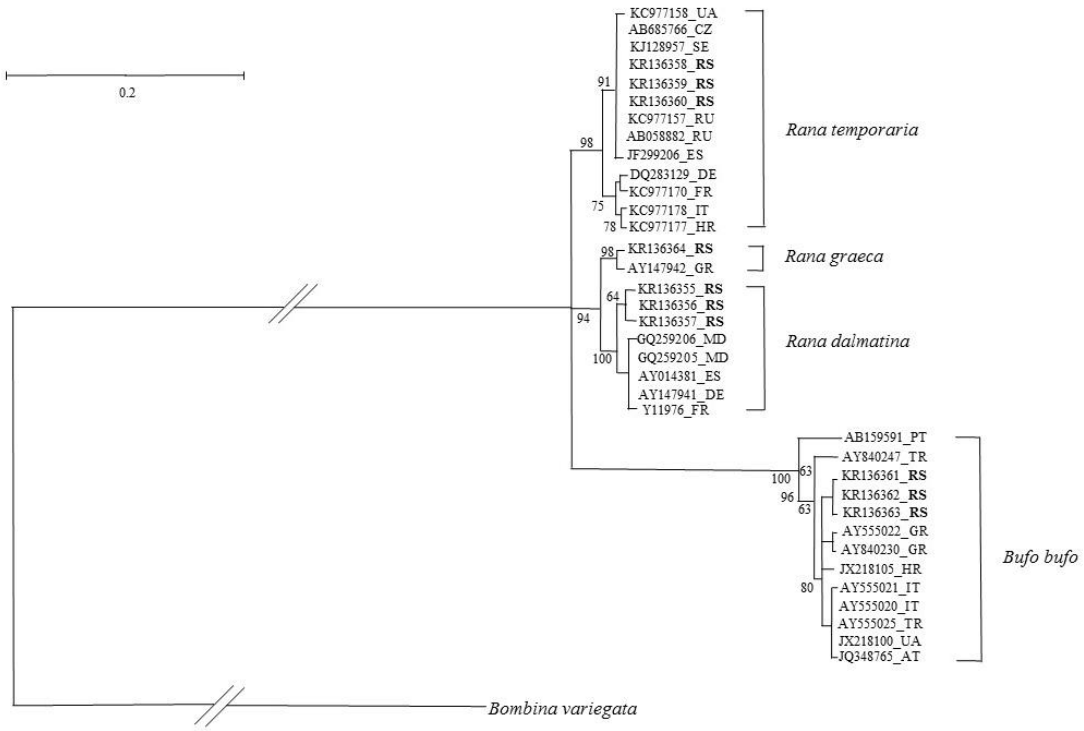

Figure 2. *Phylogenetic analysis of 16S rRNA gene fragment by Maximum Likelihood method based on the Tamura-Nei model. Bootstrap value (<60%) was showed and confirmed the clade at the end of a branch.*

Morphometric analysis

The value ranges of selected raw measurements are presented in *Table 2*.

Table 2. *Value ranges of selected raw body measurements; n –sample size. Abbreviations of the variables are explained in Material and Methods. All the measurements were in mm.*

Species (n)	bl	hl	hw	tl	th
R. dalmatina (10)	10.08 – 11.27	3.47 – 3.90	1.60 – 2.05	5.92 – 7.29	1.64 – 2.46
R. temporaria (10)	8.90 – 10.68	2.75 – 4.20	1.32 – 1.84	4.91 – 6.27	1.38 – 1.94
B. bufo (10)	10.12 – 11.20	4.04 – 4.70	2.15 – 2.64	5.61 – 6.44	1.77 – 2.17

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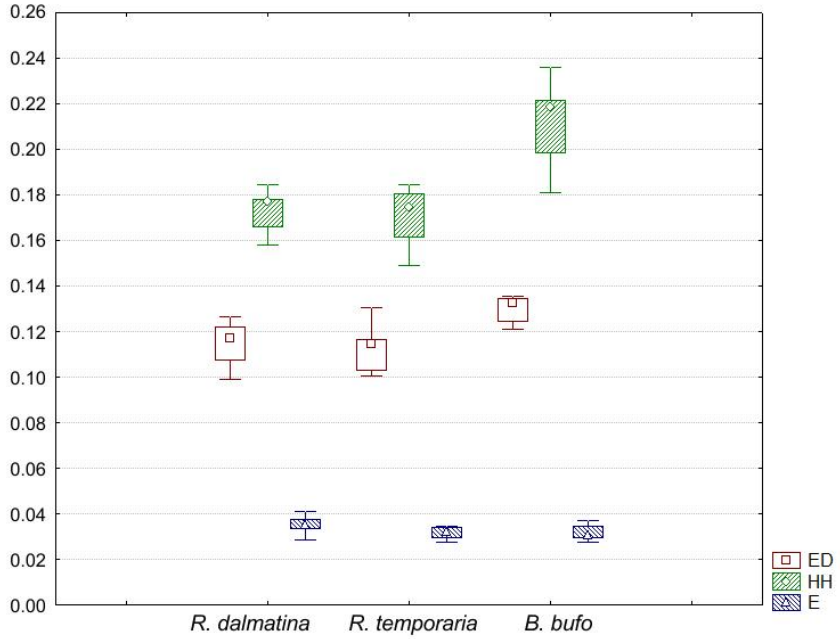
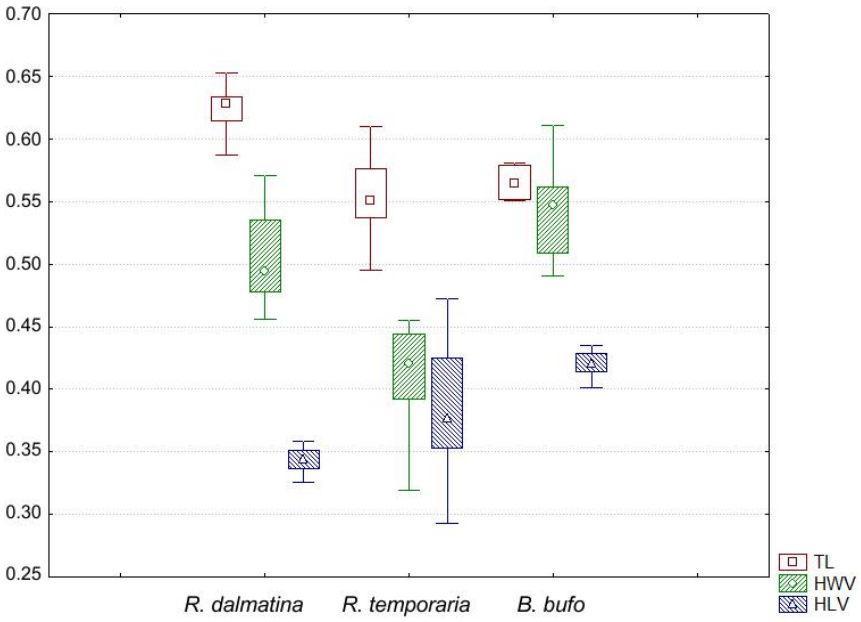
The measurements were further standardized by body length (bl) to obtain values for subsequent analyses (ED, HH, E, TL, CC, TH, M, HWV and HLV). Since the sample size was less than 50, Shapiro-Wilk's test was used to check the normality of data. In the tested data set, the obtained p-value was greater than the chosen alpha level (α=0.05), confirming that the analyzed data had a normal distribution.

The results of descriptive statistics (*Fig. 3.*) highlighted the similarity of ED and HH values among the analyzed *Rana* species, compared to *B. bufo*, suggesting that these two parameters could distinguish the two genera at early tadpole stages.

a)



b)



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c)

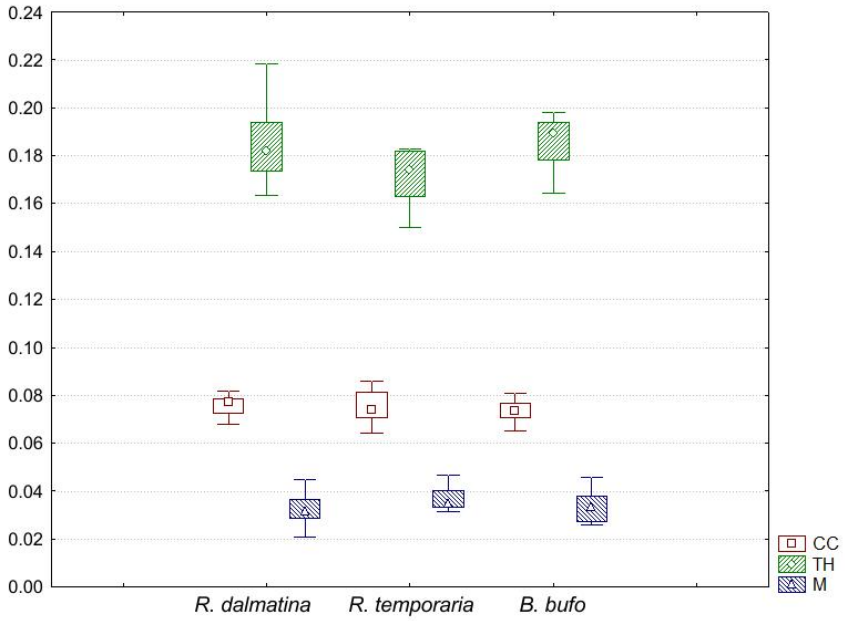

Figure 3. *Descriptive statistics of the standardized measurements in tadpoles of analyzed anuran species: a) variable ED, HH and E; b) variable TL, HWV and HVL; c) variable CC, TH and M. Abbreviations of the variables are explained in Material and Methods.*

Moreover, TL, HLV and especially HWV differed in all three analyzed data sets. Thus, these parameters could be used in species recognition. It was notable that E, TH, CC and M values were relatively similar in all three data sets.

Performed tests of homogeneity (Levene's test and Box M test) showed homogeneity (equality of variances/covariances) for all tested variables with the exception of variable HLV.

To test statistical significance of differences between means of used variables (for analyzed species), one-way ANOVA with contrasts was applied. As it could be seen from *Table 3*, variables which differ significantly ($p<0.05$) among examined species, were ED, HH, TL, HWV and HLV.

Table 3*. Results of one –way ANOVA with contrasts (significant differences (p<0.05) are in bold); Estimate –Estimated mean value; Std. Err –Standard Error; t – t value; p – p value; Cnf. Lmt (-95%), Cnf. Lmt (+95%) – Confidence Intervals for Mean.*

	Estimate	Std. Err	t	p	Cnf. Lmt (-95%)	Cnf. Lmt (+95%)
ED	-0.032690	0.006351	-5.14736	**0.000021**	-0.045721	-0.019659
HH	-0.085195	0.011152	-7.63957	**0.000000**	-0.108077	-0.062314
E	0.003240	0.003502	0.925069	0.363125	-0.003946	0.010425
TL	0.047562	0.017595	2.703096	**0.011736**	0.011459	0.083665
CC	0.005678	0.004581	1.239504	0.225825	-0.003721	0.015078
TH	-0.016355	0.010192	-1.60466	0.120203	-0.037267	0.004558
M	0.002175	0.005979	0.363826	0.718821	-0.010092	0.014443
HWV	-0.156075	0.037526	-4.15910	**0.000290**	-0.233073	-0.079078
HLV	-0.106239	0.025620	-4.14680	**0.000300**	-0.158806	-0.053672

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Prior to applying multivariate analysis, Pearson's product-moment correlation was run to eliminate uncorrelated parameters. Eye diameter (E), central tail muscle (CC) and mouth length (M) showed no correlation ($p<0.05$) and were thus excluded from further analysis (*Table 4*).

Table 4. *Pearson correlations among tested variables (marked correlations that are significant at $p < 0.05$ are in bold). Abbreviations of the variables are explained in Material and Methods.*

	ED	**HH**	**E**	**TL**	**CC**	**TH**	**M**	**HWV**	**HLV**
ED	1.000	**0.450**	-0.047	-0.314	-0.244	0.065	0.135	0.320	**0.413**
HH	**0.450**	1.000	0.004	-0.225	-0.114	**0.365**	0.091	**0.576**	**0.453**
E	-0.047	0.004	1.000	0.121	0.139	-0.028	-0.344	-0.110	0.074
TL	-0.314	-0.225	0.121	1.000	0.229	0.300	-0.217	0.211	**-0.384**
CC	-0.244	-0.114	0.139	0.229	1.000	0.113	0.142	0.014	-0.005
TH	0.065	**0.365**	-0.028	0.300	0.113	1.000	0.087	**0.521**	-0.137
M	0.135	0.091	-0.344	-0.217	0.142	0.087	1.000	-0.077	0.088
HWV	0.320	**0.576**	-0.110	0.211	0.014	**0.521**	-0.077	1.000	-0.226
HLV	**0.413**	**0.453**	0.074	**-0.384**	-0.005	-0.137	0.088	-0.226	1.000

Canonical Discriminant Analysis (CDA) revealed that HWV and HLV are the most informative characters for taxonomical distinction between tadpoles of brown frogs and those of the common toad, while TL was considered the most informative for distinguishing between tadpoles of two brown frog species.

Table 5. *The roots of the discriminant analysis (CDA), their discriminatory power and loads (the most discriminative variables are in bold).*

	Root 1	**Root 2**
ED	-0.31469	0.480344
HH	-0.27051	-0.075231
TL	-0.19050	**1.049517**
TH	-0.11888	0.113444
HWV	**-0.66539**	0.068916
HLV	**-0.83712**	-0.426270
Eigenval	12.58955	3.311605
Cum.Prop	0.79174	1.000000

The complex relationship among selected morphometric parameters in analyzed tadpole samples was presented in two-dimensional space of first and second discriminant axes (*Fig. 4.*).

The first axis (root) distinguished *B. bufo* (to the left of the graph) from genus *Rana* (to the right of the graph). Distinction of the analyzed *Rana* species occurred along the second root.

Linear regression analysis was used for a detailed investigation of the relationship between the most significant variables, by canonical discriminant analyses (HLV, HWV

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and TL). As values of the coefficient of determination (r^2) were low (< 0.3), only the TL/HWV relationship was presented on the graph (*Fig. 5*).

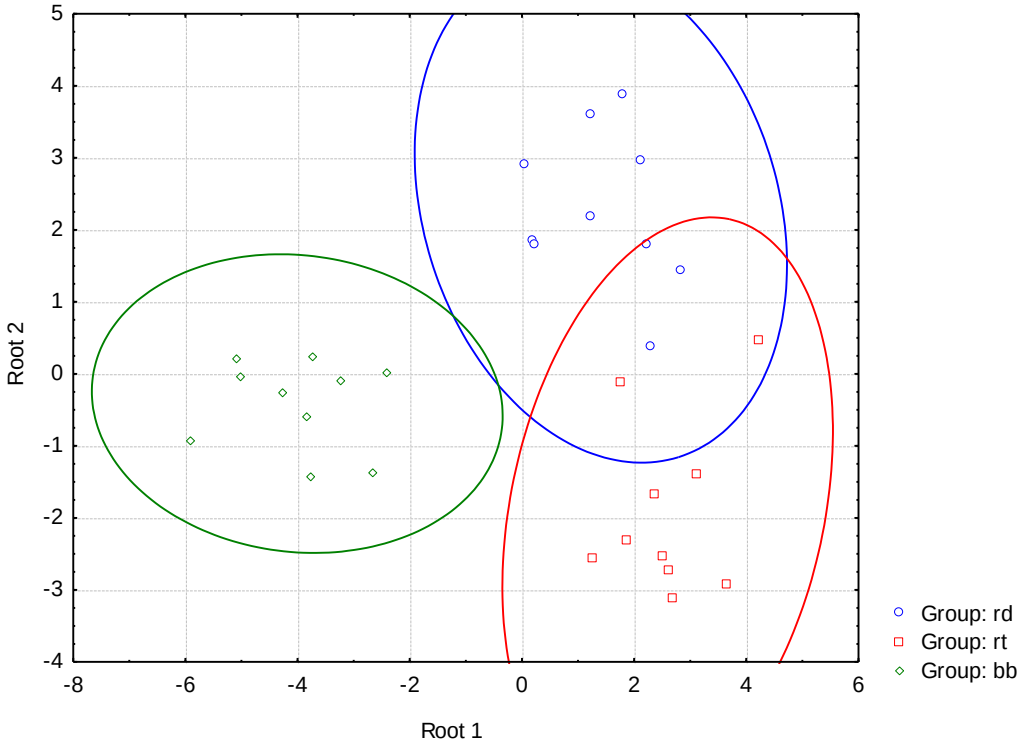

Figure 4. *Canonical Discriminant Analysis: rd – R. dalmatina, rt – R. temporaria, bb – B. bufo.*

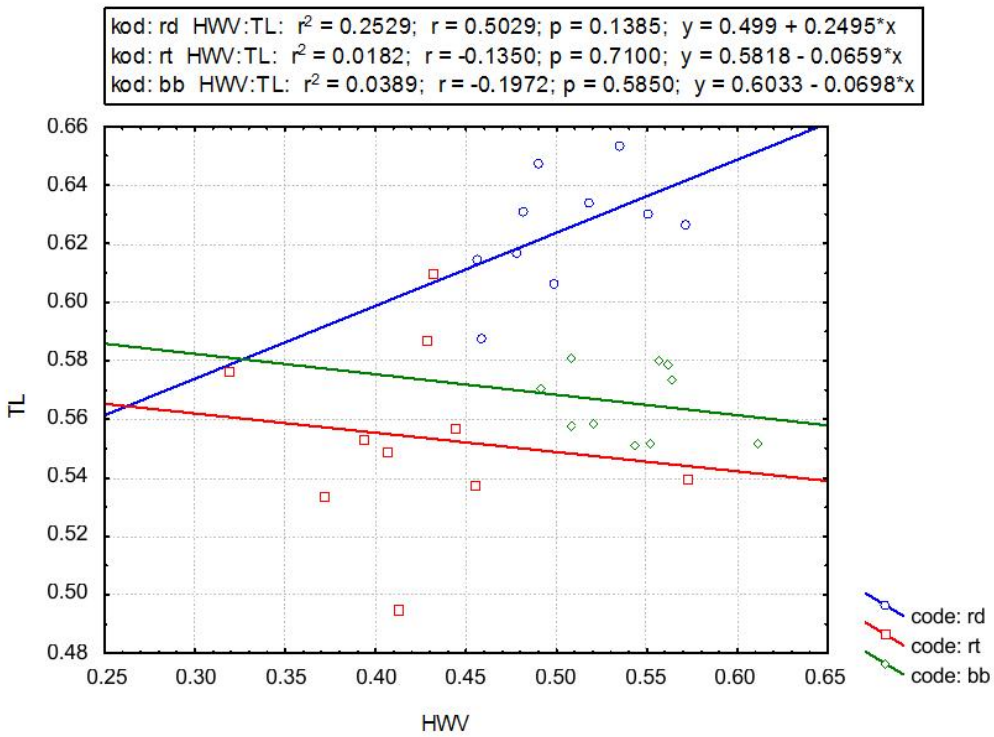

Figure 5. *Regression analysis of head width (HWV) on tail length (TL); rd – R. dalmatina, rt – R. temporaria, bb – B. bufo.*

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It was notable that *R. dalmatina* showed a positive correlation, in contrast to the negative one occurring in *R. temporaria* and *B. bufo*. The best approximation with a linear regression line was observed in *R. dalmatina* (r^2=0.25), while the weakest one was detected in *R. temporaria* (coefficient of determination was 0.01).

Discussion

Gene sequence, based on *16S rRNA* gene, confirmed the morphological identification of the analyzed species. The obtained phylogenetic tree showed that the *R. temporaria* clade was the most heterogeneous, as mentioned previously in the work of Reh and Seitz (1990) and Vences et al. (2013). Further, analyzed sequences from Serbia were shown to be closer to the North-Eastern group (samples form Czech Republic, Russia, Ukraine and Sweden) than to specimens from the South and Western group (samples from Croatia and Italy). A similar relation was detected in another typical boreal species e.g. *Zootoca vivipara* (ex *Lacerta vivipara*), where Surget-Groba et al. (2001) showed a closer similarity between Bulgarian populations and East European ones than with adjacent parts of the Balkan Peninsula and Western Europe (Crnobrnja-Isailović, 2007). Although the common frogs' postglacial re-colonization of Europe was not as straightforward as had been previously assumed, with numerous small, cryptic refugia (Teacher et al., 2009), our results could denote Serbia as one of these refugia (or re-colonization hotspots) in the case of North-East Europe.

R. dalmatina samples from Serbia were well separated from others collected in Spain, Moldova, France and Germany. In the *B. bufo* clade, samples from Serbia were placed together with those from Greece, Italy, Austria and Croatia, but somewhat apart from the sample from Portugal, being in line with the results of Recuero et al. (2012) who revealed that common toads from the Iberian Peninsula belong to *B. spinosus*, while Appenine, Central European and Balkan ones represent *B. bufo.*

Descriptive statistics and one-way ANOVA showed fewer differences in samples of *R. dalmatina* and *R. temporaria* tadpoles in comparison with *B. bufo*. Canonical Discriminant Analysis showed that the most useful measures for distinguishing tadpoles of the three analyzed species were body size standardized tail height (TL), head width (HWV) and head length (HLV). Relative head size is a determinative characteristic for distinguishing between *Rana sp.* and *B. bufo*, while the relative tail length is the most informative for distinguishing between the two *Rana* species. Additionally, in all cases linear regression analysis of the most informative variables (HLV, HWV and TL) showed a separation of *R. dalmatina* from the other two analyzed species, while the coefficient of determination (r^2) was low in all cases.

Furthermore, we learned from this study that *B. bufo* and *R. graeca* tadpoles could be visually misidentified at very early developmental stages if they occur syntopically in highland running waters in the early spring (a common spawning type for the Greek frog but not so usual for the Common toad). Our further analysis will include comparison of the same morphometric measures used in this study, taken simultaneously from tadpoles of all three brown frog species and the common toad, all raised in the laboratory from fertilized eggs collected in the natural habitat in order to follow, compare and detect significant allometric changes occuring within and among three species. Additionally, our future plans include also a comparative body shape analysis on samples of three species of brown frogs and the common toad, done at several stages of tadpole development by using geometric morphometrics.

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APPENDIX

Appendix 1. *The list of sequences that were used in the analyses (GenBank accession numbers and countries of origin).*

Species	Country	Access no.
Rana dalmatina	Serbia	KR136355
Rana dalmatina	Serbia	KR136356
Rana dalmatina	Serbia	KR136357
Rana dalmatina	Germany	AY147941
Rana dalmatina	France	Y11976
Rana dalmatina	Spain	AY014381
Rana dalmatina	Moldova	GQ259206
Rana dalmatina	Moldova	GQ259205
Rana graeca	Serbia	KR136364
Rana graeca	Greece	AY147942
Rana temporaria	Serbia	KR136358
Rana temporaria	Serbia	KR136359
Rana temporaria	Serbia	KR136360
Rana temporaria	Spain	JF299206
Rana temporaria	Germany	DQ283129
Rana temporaria	France	KC977170
Rana temporaria	Russia	AB058882
Rana temporaria	Russia	KC977157
Rana temporaria	Sweden	KJ128957
Rana temporaria	Ukraine	KC977158
Rana temporaria	Czech Republic	AB685766
Rana temporaria	Italy	KC977178
Rana temporaria	Croatia	KC977177
Bufo bufo	Serbia	KR136361
Bufo bufo	Serbia	KR136362
Bufo bufo	Serbia	KR136363
Bufo bufo	Portugal	AB159591
Bufo bufo	Italy	AY555021
Bufo bufo	Italy	AY555020
Bufo bufo	Turkey	AY840247
Bufo bufo	Turkey	AY555025
Bufo bufo	Greece	AY555022
Bufo bufo	Greece	AY840230
Bufo bufo	Croatia	JX218105
Bufo bufo	Ukraine	JX218100
Bufo bufo	Austria	JQ348765
Bombina variegata	HE794027	

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