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Tadpole morphology of two spadefoot toads (*Pelobates fuscus* and *P. syriacus*)

(Amphibia, Anura, Pelobatidae)

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Intraspecific variation in body mass and morphometric and qualitative characters of different larval stages were studied in a number of population samples of *Pelobates fuscus* and *P. syriacus* from Serbia and Macedonia. Tadpoles were collected from populations within the areas of allopatry and sympatry, in both allotopic and syntopic locations. It was discovered that, on the average, larval body size increased linearly to stage 41, and then suddenly decreased in the last stages. Changes in size and shape relationships, studied by Principal Component Analysis, revealed that interspecific differences appeared to be much larger between younger tadpoles, with the main difference being in head size measurements. Convergence in morphometric aspect progressed towards the time of metamorphosis. Canonical Discriminant Analysis revealed slight species differences on the basis of tadpole morphometric characteristics, including body mass. This was found for both pooled samples of all stages analysed, as well as for particular larval stages. However, on the whole, the effect of locality was apparently very influential. Studied qualitative traits failed to discern any geographic trends or provide a ready means to segregate species among the different localities. Consequently, it can be inferred that tadpole morphological characters can not be used to distinguish between the two species except in early growth stages.

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Introduction

The spadefoot toads of the genus *Pelobates* include four species, among them the common spadefoot *(P. fuscus)* and the eastern spadefoot *(P. syriacus)*. These two toads are clearly differentiated species. They belong to two different phylogenetic lineages within the genus *Pelobates* (Maglia 1998). With respect to their distribution, *P. fuscus* has an extensive range which covers most of the plains and hilly steppic regions of Central, Eastern and Southeastern Europe (Nollert 1997). The eastern spadefoot (*P. syriacus*) has a range extending from the southern part of the Panonian Plain (Banat) through Southeastern Romania, Bulgaria, and Greece to Southwestern Asia (Sofianidou 1997). *P. fuscus* and *P. syriacus* have narrow zones of sympatry. Today these two species occur together along the lower course of the Danube, as well as in the vicinity of the Bosphorus to the south (Dzukic & Pasuljevic 1983, Eiselt 1988).

Adult specimens of *P. fuscus* and *P. syriacus* can be easily distinguished due to pronounced differences in coloration patterns, as well as by the presence *(P. fuscus)* or absence *(P. syriacus)* of a well-marked dome on the top of the head. Also, adult individuals of *P. syriacus* are on the average, much larger than those of *P. fuscus*, including within the area of their sympatry (Rot-Nikcevic et al. 2001). These two species also differ in the direction of the magnitude of sexual size dimorphism. *P. fuscus* follows the general trend in amphibian species, having much larger females than males, while in *P. syriacus* sexes have approximately the same body size, or the males can be larger than females in some cases (Rot-Nikcevic et al. 2001). On the other hand, studies of life-history traits showed that interspecific differences in longevity and time of attainment of sexual maturity appeared to be small and without a consistent pattern of variation.

Tadpoles of all European anuran congeneric species can be distinguished by their external morphological traits, except for green frogs (*R. esculenta* complex) and spadefoot toads. The search for possible diagnostic morphological traits for their tadpoles is of importance in the area of species sympatry, especially in the sites of syntopy (i.e. where adults of both species breed concurrently).

To the best of our knowledge, an in-depth morphological analysis of *Pelobates* tadpoles, especially intra- and interpopulation variation patterns, has never been done. Current knowledge comes primarily from a few publications describing only a limited number of tadpole traits, for example body shape and coloration. In regard to interspecific comparisons, in several taxonomic keys it was claimed that tadpoles of *P. fuscus* and *P. syriacus* can not be distinguished (e.g., Engelmann et al. 1986), while Fuhn (1960) and Kuzmin (1999) pointed out some differences, mainly in coloration and body shape.

The purpose of this paper is twofold. First, an in-depth analysis of both morphometric and qualitative external morphological characteristics of different larval stages in a number of population samples of *P. fuscus* and *P. syriacus* was performed. Second, in analysing tadpoles which came from the areas of allopatry and sympatry, both allotopic and syntopic locations, this study attempts to evaluate the influence of location, stage of tadpole development and patterns of data variation for both species.

Material and methods

Population samples. Tadpoles were collected from allopatric areas: three localities for *P. fuscus* (Hrastovaca, Crepaja and Samos) and two localities for *P. syriacus* (Prdejci and Djavato), as well as from the zone of sympatry (Deliblato Sand). Within the range of species coexistence, the allotopic breeding sites of *P. fuscus*, but not of *P. syriacus*, and more numerous syntopic breeding sites were recognized. In this analysis, one allotopic population sample (Zamfir bara) and two nearby population samples with tadpoles of both species (Zubanov and Djabin salas), have been included (see Appendix for sample size and more details about localities). Samples were collected in June and July of 1999 and 2000, and preserved in 70 % ethanol. Analysed larvae were in various stages of development: from 34 to 45 (according to Gosner 1960). In order to obtain an appreciable number of larvae for our analyses, we pooled initial stages into reduced stage orders as follows: stage I (stages 34, 35 and 36); stage II (stages 37 and 38); stage III (stages 39 and 40); stage IV (stage

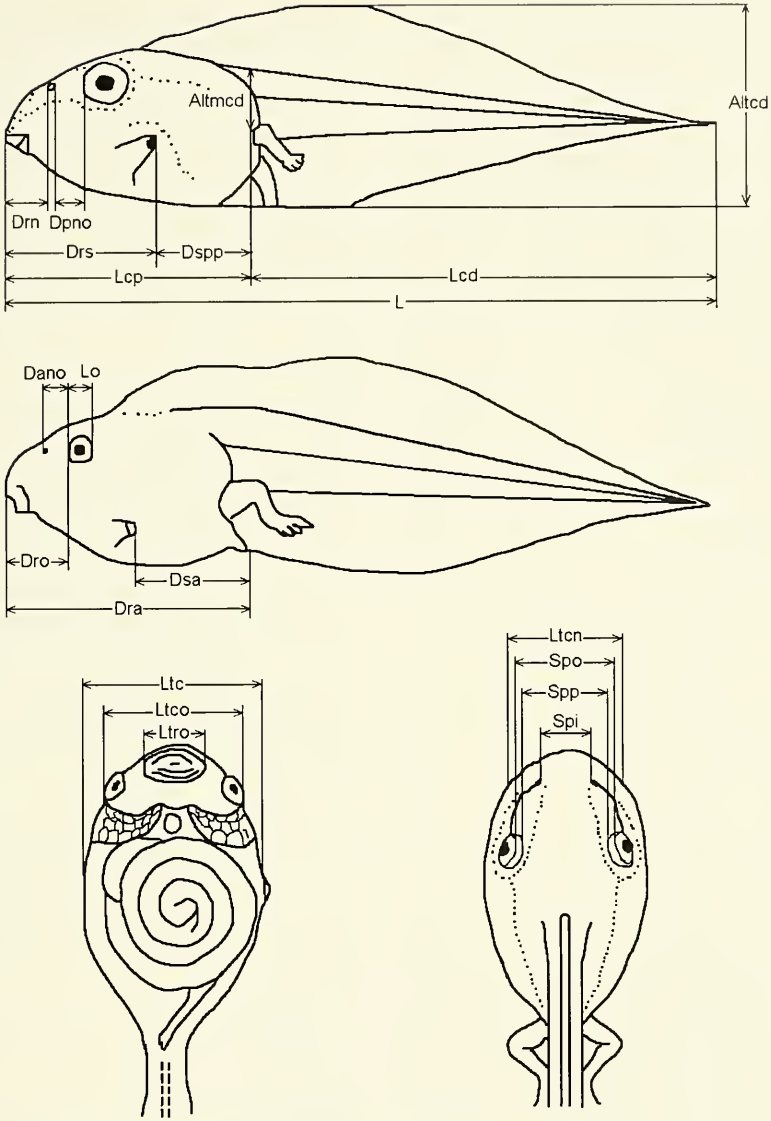

Fig. 1. Body size characters measured. For character abbreviations see text.

41); and stage V (stages 42, 43, 44 and 45). Larvae from Djavato were kept in the laboratory for a period of three months (March-June, 2000), from the blastula to tadpole stage 44. Larvae were housed in an aquarium (150 l), with various plants from their natural habitat. The aquarium was exposed to a natural photoperiod and daily changes in room temperature (15-25 °C). A constant water level was maintained. Water was continually recycled and filtered. Thus, the larvae were maintained in standard conditions with *ad libitum* access to food (boiled spinach and commercial aquarium fish food). At the beginning of June, when larvae had attained a size between stages 36 to 44, they were preserved by immersion in 70 % ethanol.

The specimens are deposited in Georg Dzukic's Batrachological collection (Institute for Biological Research, Belgrade), and in the Vertebrate collection of the Macedonian Museum of Natural History, Skopje, Macedonia.

Characters studied. We measured the following characters with a digital caliper to the nearest 0.01 mm (Fig. 1): Drn: snout to nostril distance (distance between the tip of the snout to the nasal pore), Dpno: posterior nostrils to anterior eyelid distance (distance between the anterior eyelid commissures to the posterior edge of the nasal pore), Drs: snout to spiracle distance (distance between the tip of the snout to the spiracle pore), Dspp: spiracle to hindlimbs distance (distance between the spiracle pore to the anterior margin of the hindlimb at insertion), Altmcd: tail musculature height (at insertion of hindlimbs), Lcp: body length (from the snout to the anterior margin of the hindlimb at insertion), Lcd: tail length (from the anterior margin of the hindlimb at insertion to the tail tip), L: total length (from the snout to the tail tip), Altcd: tail height (maximum tail height), Dro: snout to eye distance (distance between the tip of the snout to the anterior eyelid commissures), Dano: anterior nostrils to anterior eyelid distance (distance between the anterior eyelid commissures to the anterior edge of the nasal pore), Dra: snout to anal pore distance (distance between the tip of the snout to the posterior edge of the anal pore), Dsa: spiracle to anal pore distance (distance between the spiracle pore to the posterior edge of the anal pore), Ltro: width of mouth region, Spi: distance between nostrils, Spp: minimum interorbital distance, Spo: interocular distance (distance between the eyes), Lo: eyeball length (from the anterior to the posterior eyelid commissure), Ltcn: head width at position of nostrils, Ltco: head width at position of posterior eyelid commissure, Ltc: maximum head width, F: length of femur (from insertion to the distal end of the femur), T: length of tibia (from the proximal end of the tibia to the metatarsal wrist), N: foot length from the heel to the end of the longest toe, P: foot length from the metatarsal wrist to the end of the longest toe, Ltf: width of femur (at the widest part), Ltt: width of tibia (at the widest part), Cint: length of inner metatarsal tubercle. Body weight (M) was measured to the nearest 0.001 g.

Qualitative characters were as follows: I: Colour of body: a: golden yellow, b: olive green, c: grey, d: black; II: Presence of spots: a: present, b: not present; III: Shape of marginal papillae of mouth region: a: conical, b: pyramidal, c: low conical; IV: Constitution of tadpole body: a: stout, b: slender; V: Shape of tail tip: a: rounded, b: pointed.

Statistical procedures. In order to minimise deviations from normality caused by allometric relationships, all body measurements and body mass were log-transformed. Student's t-test was used to test differences in mean values between localities for every stage, considering $P<0.05$ as a level for significance. A one-way analysis of variance (ANOVA) was performed using population samples or stages as factors. In order to determine the degree of difference between species and among stages, a multivariate analysis of variance (MANOVA) was used. Principal Component Analysis (PCA), was used to examine patterns of morphometric variation between species through ontogenic changes in tadpoles' size and shape. The analysis was performed on the covariance matrix of log-transformed variables. Separate PC analyses were computed for five larval stages of two samples (Hrastovaca: *P. fuscus* and Prdejci: *P. syriacus*). Canonical Discriminant Analysis (CDA), of log-transformed data was used to distinguish between intraspecific and interspecific variation for morphometric measurements. To reduce the influence of body size, CDA was also performed on residuals from a regression analysis of each trait on snout to anal pore distance (Dra). Percentages of states were calculated for each qualitative trait. Programs designed and implemented by the STATISTICA (StatSoft, Inc. 1997) package were used.

Results

Morphometric characters. The largest measured *P. fuscus* tadpole came from the Samos sample, with a body length of 110.8 mm (stage III), while the minimum total length for a *P. fuscus* tadpole was recorded in the Zamfir bara sample (53.5 mm, stage I). Within *P. syriacus*, the maximum and the minimum total lengths were recorded in the Prdejci sample (104.0 mm, stage IV, and 40.8 mm, stage V). Univariate analysis of variance demonstrated high significance among sample differences for all characters (ANOVA, $P<0.001$). Moreover, there were significant differences among samples within species. Consequently, tadpoles from a pair of *P. syriacus* samples (Prdejci and

Djavato) significantly differed in 17 out of 29 characters. Among *P. fuscus* samples, a comparison of tadpoles from Hrastovaca vs. Samos and Crepaja had 21 out of 29 characters being significantly different. On the other hand, *P. fuscus* tadpoles from two geographically close samples (Samos and Crepaja) differed significantly in only 6 of 29 traits. The greatest differences among population samples appeared to be in comparisons of the first and the second larval stages (significant differences in 27 of 29 characters, and in 26 of 29 characters, respectively). However, differences were small or without significance in the third and the fourth stages with regard to hindlimb measurements (significant differences in 1 of 7 hindlimb characters, and in 2 of 7 hindlimb characters, respectively). In the fifth stage, differences became the smallest for most morphometric characteristics (14 non-significant traits), except for hindlimb measurements.

When the larval stages were considered separately, there was a tendency for increased tadpole body length (L) up to the fourth stage (stage 41 according to Gosner 1960), and a subsequent rather sharp decrease in the last stage (V – 42-45) due to drastic metamorphic changes (Table 1). On the average, the largest tadpoles at the fourth stage were *P. fuscus* from the Zamfir bara sample (100.1 ± 5.7 mm), and the smallest, *P. syriacus* tadpoles raised in the laboratory, Djavato sample (72.6 ± 1.6 mm). Taking into consideration all studied morphometric characters, paired testing of differences among samples revealed a complete departure of the Djavato sample *(P. syriacus)* at every stage. When the tadpoles of the first larval stage were compared, the greatest differences in pairwise sample comparisons appeared between Djabin salas (syntopic site) and Djavato (significant differences for 16 of 22 character comparisons). Non-significant differences were found for Zamfir bara *(P. fuscus)* vs. Zubanov salas (syntopic site); Zamfir bara vs. Prdejci (P. syriacus) and Zubanov salas vs. Djabin salas (syntopic sites) pairs. At the second stage the greatest differences existed when the Djavato sample *(P. syriacus)* was compared with the two *P. fuscus* samples (Hrastovaca and Samos, 19 out of 29), while the smallest differences appeared between two nearby *P. fuscus* samples (Samos and Crepaja, 3 out of 29). The greatest differences at the third stage were between samples from Hrastovaca and Crepaja (*P. fuscus*, 18 out of 29), while the

Tab. 1. Sample means and standard errors (SE) for total length: L (in mm) in different tadpole stages of population samples. N = sample size.

Locality	I	II	III	IV	V
	N Mean ± SE	N Mean ± SE	N Mean ± SE	N Mean ± SE	N Mean ± SE
Hrastovaca *(P. fuscus)*	8 71.49 ± 3.44	11 75.35 ± 1.80	7 78.96 ± 3.71	11 85.26 ± 1.42	4 77.69 ± 7.87
Samos *(P. fuscus)*	4 80.23 ± 1.90	6 83.56 ± 2.02	3 92.86 ± 9.01	2 89.68 ± 3.70	– –
Crepaja *(P. fuscus)*	– –	11 86.50 ± 2.80	12 96.65 ± 1.26	3 98.83 ± 1.95	– –
Zamfir bara *(P. fuscus)*	5 77.41 ± 7.01	6 79.63 ± 6.10	– –	4 100.15 ± 5.68	– –
Zubanov salas *(P. fuscus/P. syriacus)*	4 64.09 ± 1.43	– –	2 81.73 ± 0.14	3 82.45 ± 6.84	– –
Djabin salas *(P. fuscus/P. syriacus)*	13 66.63 ± 1.03	3 69.83 ± 2.63	11 73.71 ± 2.91	5 78.64 ± 2.18	3 63.01 ± 6.02
Prdejci *(P. syriacus)*	5 84.86 ± 5.57	3 87.23 ± 7.92	4 80.55 ± 8.42	7 92.32 ± 3.23	16 74.75 ± 4.02
Djavato *(P. syriacus)*	10 76.65 ± 1.59	4 72.63 ± 3.86	– –	9 72.61 ± 1.63	11 68.87 ± 1.70

samples from Samos and Zubanov salas lacked any significantly different character comparisons. The fourth stage revealed the greatest distinction between tadpoles from two *P. syriacus* samples (Prdejci vs. Djavato, 21 out of 29), while only one out of 29 character comparisons appeared to be statistically significant when Zamfir bara and Zubanov salas were compared. The testing in the fifth stage could include only four samples. The greatest differences were between two *P. syriacus* samples (15 out of 25 character comparisons), and the smallest ones were between the Djabin salas and Prdejci samples (1 out of 25).

Multivariate analysis of variance (MANOVA) with species and stage as factors revealed significant differences in snout to nostril distance (Drn, $P < 0.05$), body length (Lcp, $P < 0.01$), snout to anal pore distance (Dra, $P < 0.001$), head width at position of nostrils (Ltcn, $P < 0.01$), maximum head width (Ltc, $P < 0.05$), and hindlimb measurements (F, $P < 0.05$; T, $P < 0.01$; N, $P < 0.05$; P, $P < 0.05$; Ltf, $P < 0.05$; Ltt, $P < 0.01$).

PCA showed significant differences between *P. fuscus* and *P. syriacus* tadpoles during larval stages. There was obvious segregation on the first principal component (PC1) in the first stage. The body weight (M), head width at position of nostrils (Ltcn), maximum head width (Ltc), snout to nostril distance (Drn) and spiracle to hindlimbs distance (Dspp) were the highest-loading characters on the first component. Tadpoles of *P. syriacus* had higher relative values for these traits. Segregation in the second stage is less obvious, although there was a certain distinction along the PC2 on the basis of a greater influence from head width (Ltcn, Ltc and Ltco) in *P. syriacus* tadpoles. A tendency for differentiation on the basis of these same characters continued during the third and fourth stages where Ltcn, Ltco and Ltc were the highest-loading characters on the PC3. There were no apparent differences between species in the fifth stage (figures and additional statistical data are available from corresponding author upon request).

The Canonical Discriminant Analysis (CDA), of morphometric characters and body weight, performed on pooled population samples without regard to larval stage, revealed 82.5 % of the total variation expressed in the first three canonical axes. The mean percentage of correctly classified cases in each sample was very high (89.6 %). The smallest correctness value (66.7 %) was for Zubanov salas (syntopic site), and the greatest (100 %) from the samples Samos, Crepaja *(P. fuscus)* and Djavato *(P. syriacus)*. The first three canonical variates appeared bipolar (the table of pooled within-groups correlations between variables and canonical axes is available from corresponding author upon request). Hindlimb measurements were the reverse of the other traits on the second canonical variate. Most traits had the highest and most significant correlation on the second variate. This suggests that discrimination among samples based on this canonical variate may be caused by overall size. Differences between species were the most discernible in the region of the first and the second canonical axes (Fig. 2). Samples of *P. syriacus* and *P. fuscus* were situated on opposite sides of the second axis. Tadpoles of P. syriacus from Macedonian samples (Prdejci and Djavato) had in general, higher values for hindlimb measurements in comparison to the others. On the other hand, they had the lowest values for some other traits (Drn, Dpno, Altcd, Dro, Dano, Spi and Spp). Larvae of *P. fuscus* from the Samos and Crepaja samples, situated in the upper right quadrant, were differentiated from the remaining ones mainly on the basis of the longest and tallest tail (Lcd, Altcd) and the longest body (L), but they had the lowest mean values for all characters concerning hindlimbs. Of special note, one sample of presumably mixed *P. fuscus* and *P. syriacus* tadpoles (from Zubanov salas) appeared well in the morphospace of *P. fuscus*, while some individuals of the second sample from a syntopic site (Djabin salas) were close to *P. syriacus* tadpoles from Prdejci. CDA performed on residuals, when the size effect was eliminated, did not reveal greater distinction between species.

A separate CDA was done for the first and the fourth larval stages, since these two stages were present in all samples. In the first stage, the CDA revealed 79.1 % of the total variation expressed in the first three canonical axes. There were no misclassifications among samples (correctness of 100 %). The apparent distinction of the Djavato sample *(P. syriacus)* was due to the low mean values for the characters M, Drn, Lcd, Dano, Dro, which made the greatest contribution to variance on the first canonical axis. The Hrastovaca sample was differentiated from

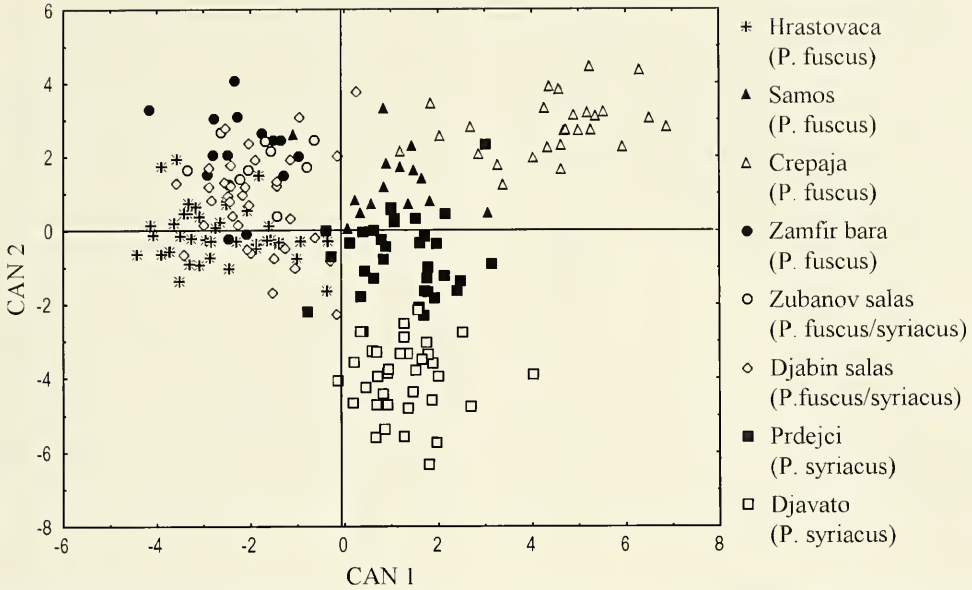

Fig. 2. Plots of canonical variable scores of the first two canonical axes (CAN) of the pooled samples of five tadpole stages.

the remaining samples on the basis of the low mean values for the characters Dra, Dsa and Drs. In the fourth stage, the CDA revealed 92.8 % of the total variation expressed in the first three canonical axes, with correct classifications of 100 %. The CDA showed a high locality effect but without any distinctive grouping of analysed population samples (figures and additional statistical data are available from corresponding author upon request).

Qualitative characters. Black body colour (Id) was predominant only in Crepaja sample *(P. fuscus)*, while grey (Ic) colour prevailed in other samples (the table of qualitative traits states percentages is available from corresponding author upon request). Tadpoles from most samples had unspotted bodies (IIb) and a mouth region with low conical papillae (IIIc). Samples of *P. fuscus* from Crepaja and in mixed samples from a syntopic locality (Djabin salas), as well as the *P. syriacus* sample from Djavato, more frequently had spotted tadpoles (IIa) than others. Tadpoles of two *P. fuscus* samples (Hrastovaca and Crepaja) and one of *P. syriacus* (Djavato) appeared to have their mouth region predominantly, with conical papillae (IIIa). In all samples, individuals with pyramidal papillae (IIIb) appeared with low frequency. Tadpoles with a slender body (IVb) and pointed tail (Vb), prevailed in most samples. Samples from one of the syntopic locations (Zubanov salas), and of *P. fuscus* (Zamfir bara) and *P. syriacus* (Prdejci) more frequently had tadpoles with stout bodies, while samples from the syntopic sites (Zubanov salas and Djabin salas) had exclusively or predominantly tadpoles with a rounded tail.

In a separate study, it was determined that the number and arrangement of keratodont rows did not differ between tadpoles of *P. fuscus* and *P. syriacus* at any larval stage studied (unpubl.).

Discussion

Tadpole body size. It is commonly understood in batrachology that spadefoot toads have the largest tadpoles in comparison to other anurans. However, except for a few scattered estimates in the literature, body size data obtained for an appreciable number of larval individuals from the same breeding site are almost non-existant. At hatching, tadpoles of *P. fuscus* and *P. syriacus* measure 4-6 mm (Kuzmin 1999). A total length of about 120 mm for *P. fuscus* individuals prior to metamorphosis in the same year of

hatching has been reported, while the multiple overwintering larvae (three years old) can reach 200 mm in total length (Grillitsch et al. 1983). To the best of our knowledge, the maximum length thus far recorded for a *P. syriacus* tadpole is 165 mm (Kuzmin 1999). Body size data for *Pelobates* tadpoles recorded from the neighbourhood of FR Yugoslavia are also rare and are based on measurements of only a few individuals. Thus, for *P. fuscus* tadpoles, Grillitsch et al. (1983) reported a total length from 80 to 120 mm (Austria), up to 100 mm for tadpoles in Hungary (Dely 1967), and 95.0 mm for tadpoles in Romania (Fuhn 1960). *P. syriacus* tadpoles from Greece range in total size from 90 to 115 mm (mean value 101.5 mm, Sofianidou 1977). From Turkey a size of 137 mm has been recorded (Zaloglu 1964). Apparently, the body size for spadefoot toad tadpoles observed in this study is well within the range of *P. fuscus* and *P. syriacus* so far recorded.

Body size and shape changes during ontogeny. On the basis of morphology, dissimilarities in body size are apparent between *P. fuscus* and *P. syriacus* adults. Interestingly enough, spadefoot toads of these two species start terrestrial life at approximately the same body size, but diverge soon during the first year of their juvenile phase (Rot-Nikcevic et al. 2001). Specifically, juveniles of *P. syriacus* appeared to have a significantly higher growth rate than that of *P. fuscus*, at least in the zone of sympatry. Also, the larger adult size for some *P. syriacus* individuals is the result of continued growth after the attainment of sexual maturity (the age at maturity ranges from two to four years; Rot-Nikcevic et al. 2001), which is not the case for *P. fuscus* toads.

During the larval aquatic phase, differences among analysed samples of *P. fuscus* and *P. syriacus* followed the opposite trend. Changes in size and shape relationships, revealed that interspecific differences appeared to be much larger between younger tadpoles, attributable predominantly to head size measurements, while the maximum convergence in morphometric respect progressed towards the time of metamorphosis.

Slight species differences on the basis of the tadpoles' morphometric characteristics, including body mass, were found for pooled samples of all stages analysed, as well as for particular larval stages. However, in the overall pattern of sample differentiation, the effect of locality was apparently very influential. Thus, two *P. syriacus* samples in which individuals were grown under quite differing conditions (tadpoles from Prdejci were collected from nature while those of Djavato were raised in an aquarium) differed substantially in spite of the close geographical proximity of their habitats, which does not exclude a possible gene-flow between populations. Tadpoles of *P. fuscus* also experience a great site influence in the shaping of morphometrics, to the extent that no misclassifications among samples were observed in some cases. Individuals of one sample from a syntopic site, with tadpoles of both species, were placed mainly within the *P. fuscus* morphospace, while some individuals of another syntopic sample appeared to be closer to one *P. syriacus* sample.

In summary, from these results it can be inferred that multivariate morphometrics of tadpoles, as well as the study of their integumental qualitative traits (which failed to discern any geographic trends or segregation of species among the different localities), can not discriminate between spadefoot toad species, especially those from the zone of sympatry. Thus it can not be used in the process of species identification, except in very early stages. The use of molecular techniques, such as genetic markers of allozyme loci, would need to be used to assess the specific status of premetamorphosed individuals of *P. fuscus* and *P. syriacus*.

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Appendix

Localities of population samples, their altitudes, UTM code (10 × 10 km^2), and the sample size (numbers of tadpoles in larval stages I+II+III+IV+V).

1. Hrastovaca (Vojvodina, Serbia, 110 m above sea level, CS 91, 8+11+7+11+4)
2. Samos (Vojvodina, Serbia, 120 m, DR 80, 4+6+3+2+0)
3. Crepaja (Vojvodina, Serbia, 95 m, DQ 78, 1+11+12+3+0)
4. Zamfir bara – Deliblato Sand (Vojvodina, Serbia, 150 m, EQ 16, 5+6+0+4+0)
5. Zubanov salas – Deliblato Sand (Vojvodina, Serbia, 75 m, EQ 26, 4+0+2+3+0)
6. Djabin salas – Deliblato Sand (Vojvodina, Serbia, 75 m, EQ 26, 13+3+11+5+3)
7. Prdejci (FYR Macedonia, 66 m, FL 26, 5+3+4+7+16)
8. Djavato (FYR Macedonia, 66 m, FL 26, 10+4+1+9+11)