

Interspecific and intraspecific size and shape variation in skull of two closely related species *Bufo bufo* (Linnaeus, 1758) and *Bufo verrucosissimus* (Pallas, 1814) from Turkey

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Abstract: Anurans are the most suitable vertebrate group to see the development and also evolution in the skull, compared to other terrestrial vertebrates as they show highly derived morphology. For this reason, the cranium of anurans has been widely used for analyzing e.g., development and integration, evolutionary history, phylogenetic relationships, sexual dimorphism; and for taxon determination. We applied geometric morphometric techniques and then multivariate statistical analyses to see the skull size and shape variations in closely related *Bufo* species (*B. bufo* and *B. verrucosissimus*) inhabiting Turkey. Females have larger skull than males for both species. Ventral skull size of males differed between species and within species (among populations) whereas dorsal skulls differed only among populations. In females, only size of ventral skull side differs only among populations. Dorsal skull shape differs between the species in male individuals, while in females both dorsal and ventral skulls show a significant variation among populations. *Bufo bufo* had larger squamosal than *B. verrucosissimus*, whereas *B. verrucosissimus* had longer maxilla but shorter occipital region than *B. bufo* for both males and females. Under the control of size, the shape of skull does not differ between species for both males and females. Due to structural and functional constraints because of having similar biological and physical properties of skeletal and muscle tissues, living in similar environments or shared evolutionary history, the size and shape of skulls are found to be similar between *B. bufo* and *B. verrucosissimus* in present study. However, in order to better reveal the skull variations between these two closely related species, more detailed studies with more samples on their morphology and ecology are required.

Key words: Skull, cranial size, cranial shape, *B. bufo*, *B. verrucosissimus*, Anatolia

1. Introduction

Studies on the anatomical structures of organisms have been the main subject of biology for centuries (Adams et al., 2004). Many biological studies, concerning the classification of organisms, evolutionary biology and sexual dimorphism, are based on the definition and comparison of morphological structures, as morphology provides information on how developmental, functional and environmental pressures can affect the organism (Bardua et al., 2018). Morphometrics is the study of shape variation and its covariation with other variables (Bookstein, 1992; Dryden and Mardia, 1998). Two types of morphometric techniques are commonly used, including traditional and geometric morphometrics. Geometric morphometric methods are particularly found to be useful in detecting morphological differences especially below the species level (Loy, 1996; Adams et al., 2004; Malekian et al., 2019). In this way, phenotypic differentiations at species and even subspecies level can be evaluated using landmark-based

geometric morphometrics with a high discriminatory power (Bookstein, 1992; Rohlf and Marcus, 1993; Nolte and Sheets, 2005).

The cranial skeleton provides an excellent model system for evolutionary studies as this complex morphological structure is composed of multiple developmental and/or functional modules, which interact to both cover the head organs and fulfil various biological functions, such as foraging, respiration, vision, and olfaction (e.g., Hanken and Hall, 1993). On the one hand these modules are independent units (due to function, structure or developmental origin), but on the other hand the different modules have to cooperate to form the skull. Therefore, modules could either constrain or facilitate evolutionary change and, in turn, affect both the direction and the rate of phenotypic change (Zelditch and Caramichael, 1989; Raff, 1996; Bastir and Rosas, 2005).

Since it plays an important role in the protection of the brain, the perception of the environment (seeing, hearing,

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and smelling), and food acquisition and processing, the vertebrate skull is a functionally and developmentally complex morphological structure. For this reason, variation in morphological structure of the skull was considered significant for adaptation (Ivanović et al., 2013). On the other hand, it is frequently accepted as phylogenetically more conserved (Caumul and Polly, 2005; Vidal-Garcia and Keogh, 2017). Due to dorso-ventral flattening, most of variation of skull shape in amphibians could be captured by only its dorsal and ventral views, and these are referred to as dorsal cranium (DC) and ventral cranium (VC) (Ivanović et al., 2008). The skull is a particularly informative osteological structure to reveal the anuran behavior and ecology (e.g., Jared et al., 2005; Senevirathne et al., 2006). Anurans, with high-derived morphology, are the most suitable vertebrate group to see how the skull develops and evolve (Hall, 2008). Skulls of many anuran species exhibit biphasic development as in their life that includes larval and adult stages (Hanken and Summers, 1988; Rose and Reiss, 1993). For this reason, the cranium of anurans has been widely used for analyzing e.g. development and integration, evolutionary history, phylogenetic relationships, sexual dimorphism; and for

taxon determination (Larson, 2000; Mendelson et al., 2000; Vera and Ponssa, 2004; Fabrezi, 2006; Harrington et al., 2013; Cvijanović et al., 2017; Yıldırım and Kaya, 2017; Vukov et al., 2018).

In the western Palearctic region, the common toad species group includes four species, namely *Bufo bufo* (Linnaeus, 1758), *Bufo eichwaldi* Litvinchuk, Borkin, Skorinov and Rosanov, 2008, *Bufo spinosus* Daudin, 1803 and *Bufo verrucosissimus* (Pallas, 1814) (Arntzen et al., 2013). The Common toad, *Bufo bufo* and the Caucasian toad, *Bufo verrucosissimus* are known in Turkey (Özdemir et al., 2020). The distribution ranges of both species have been blurred in the literature for years. According to the newest paper published by Özdemir et al. (2020) based on morphological and molecular data, while *B. verrucosissimus* is delimited in Mediterranean Region at the south and Artvin province at the north of Anatolia, *B. bufo* has a wide range distribution including the Black Sea (except Artvin), Marmara and Aegean Regions (see Figure 1).

In this study, we tried to reveal interspecific and intraspecific variation in skull morphology of *B. bufo* and *B. verrucosissimus* by using geometric morphometrics.

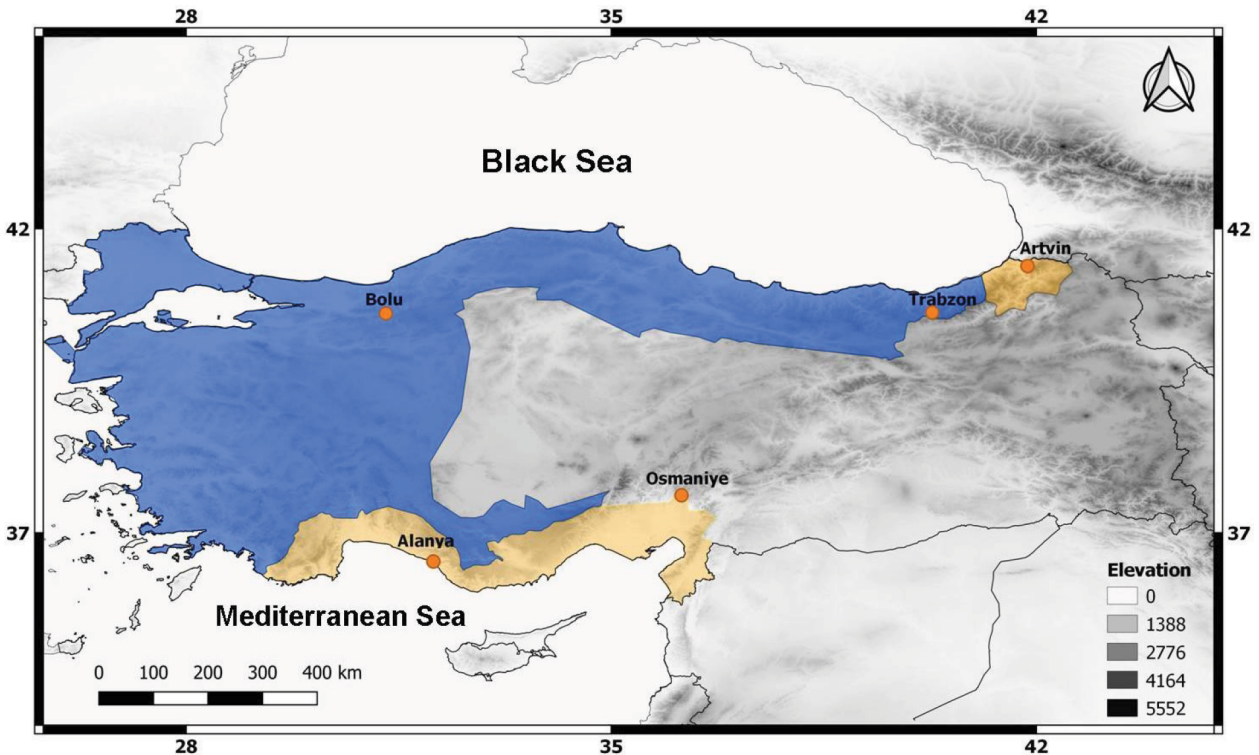


Figure 1. Distribution map of *Bufo bufo* (blue) and *Bufo verrucosissimus* (yellow) in Turkey according to IUCN Red List and Özdemir et al. (2020), and geographical positions of analysed populations.

Specifically, our aim is to determine the skull shape and size variation in these two closely related species by using a geometric morphometric approach and multivariate statistical analysis. While applying these techniques, we also emphasized intraspecific sexual differences.

2. Material and methods

2.1. Samples

A total of 48 skulls (20 specimens from 2 populations of *B. bufo* and 28 specimens from 3 populations of *B. verrucosissimus*) were examined in this study. The populations of the samples were determined according to the molecular results of Özdemir et al. (2020). The localities of the samples which were used for geometric morphometrics were given in the Figure 1.

Skulls were obtained from only adult individuals which have well-developed gonads and secondary sexual characteristics. All samples were kept in the collection of the Department of Biology at Aydın Adnan Menderes University, Aydın, Turkey.

2.2. Preparation of skulls and determination of landmarks

Skulls of all samples were first cleaned by using trypsin and potassium hydroxide. They were then stained with Alizarin red S for better visualization of the skull fragments and articulations (Digenkors and Uhler, 1977) and then kept in glycerol (Ivanović et al., 2012, 2013; Üzüüm et al., 2015). The high resolution digital camera images (Nikon D80, 3872 × 2592 pixel resolution) of the dorsal and ventral craniums were taken by positioning the palate bones and skull roof parallel to the photographic plane. To reduce and equalize distortion, the skulls were placed in the centre of the optical field (Ivanović et al., 2012, 2013; Üzüüm et al., 2015). All the photos were then transferred to the computer. Seventeen two-dimensional landmarks were digitized on both dorsal and ventral skull sides by using TpsDig2 software (Rohlf, 2008). Landmarks were digitized on the right side of each cranium by Nazan Üzüüm. All landmarks, their positions and definitions were given in Figure 2.

2.3. Statistical analysis

Because of sexual dimorphism in skull morphology, the sexes were treated separately in all analyses. Standard geometric morphometric approaches were applied based on landmark coordinates to analyze variation in the size and shape of dorsal and ventral skull. The geometric measure of the skull size was calculated as the centroid size (CS), a measure of the distribution of landmarks around the centroid of the landmark configuration (Bookstein, 1992; Zelditch et al., 2012). To explore variation in skull size, the CS was calculated and scaled according to the scale provided for each of individual skulls by using CoordGene6 software (IMP software)¹. Analysis of variance (ANOVA) was also used to determine skull size

¹ Sheets HD (2000). Integrated Morphometrics Package (IMP) [online]. Website <http://www3.canisius.edu/~sheets/morphsoft.html>.

differences (Ivanović et al., 2012, 2013; Üzüüm et al., 2015). Thin-plate spline analysis was used to obtain skull shape variables, which were used to investigate dorsal and ventral skull shape variation (Bookstein, 1989). The landmark configurations were superimposed to determine variation in skull shape by a generalized Procrustes analysis. Within analyzed sample, the CoordGen6 program was used to obtain matrix of Procrustes coordinates and the PCA analysis was performed to reveal variation in skull shape (Üzüüm et al., 2015).

In order to find out whether the species differ in skull shape, firstly a MANOVA (multivariate analysis of variance) was performed using the species as a factor. Then, by controlling the size effect, a MANCOVA (multivariate analysis of covariance) was performed, taking shape variables as dependent variables, species as factors, and CS as a covariate (Üzüüm et al., 2015). In this study, MorphoJ software was used for all geometric morphometric analyzes and visualizations (Klingenberg, 2011), and STATISTICA 7.0 (StatSoft Inc., Tulsa, OK, USA) was used for all statistical analyzes.

3. Results

Females in both *Bufo* species have larger skulls than males on both dorsal and ventral sides. Dorsal and ventral skull size data were given in Table 1. ANOVA was performed with species and populations nested within species as factors for each sex separately. In this way variation in the dorsal and ventral skull side were analyzed. ANOVA results also showed that ventral skull of males differed between species and within species (among populations) whereas dorsal skulls differed only among populations. In females, only ventral side of skull differs only among populations (Table 2).

MANOVA analysis was performed with species and populations nested within species as factors for each sex separately to analyze differences in the dorsal and ventral skull shape (Table 3). The MANOVA results indicate that dorsal skull shape differs between the species in male individuals, while in females both dorsal and ventral skulls show a significant variation among populations.

The first two PC axes which were obtained from dorsal and ventral skull shape variables and defined the positions of *B. bufo* and *B. verrucosissimus* in the shape space show that these two species largely discriminated for both sexes although the dorsal skull of male individuals appears to be partially overlapped (Figure 3). Similarly, the Procrustes distances (PD) measured between both species for both dorsal and ventral craniums were found to be statistically significant as well (Males DC, PD = 0.0616, P = 0.0019; Females DC, PD = 0.0479, P = 0.0004; Males VC, PD = 0.0643, P < 0.0001; Females VC, PD = 0.0486, P = 0.0002).

When the shapes of the dorsal and ventral skulls of the male and female individuals of *B. bufo* and *B.*

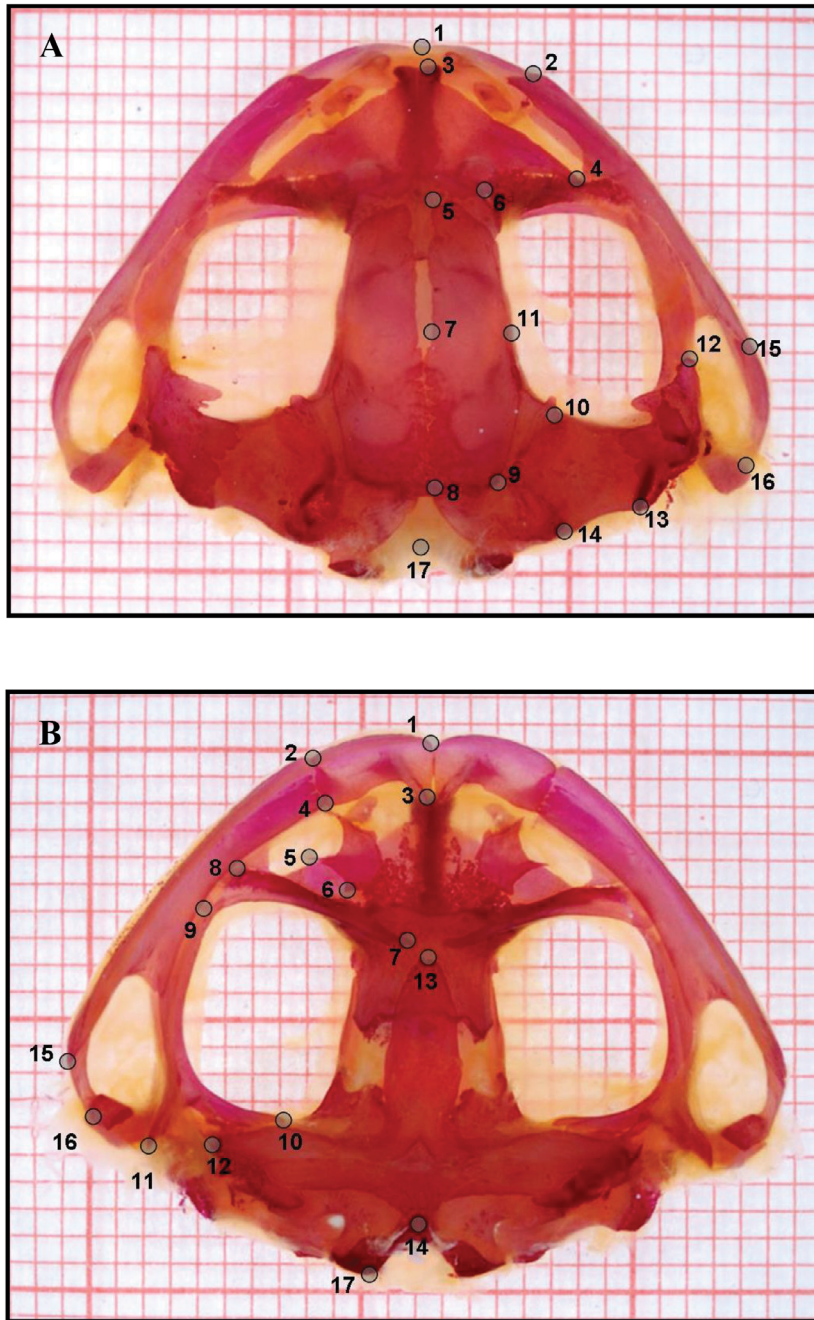


Figure 2. Location of 17 two-dimensional landmarks on the dorsal (A) and ventral (B) skull side of a common toad. A. Dorsal side: 1. Snout tip, 2. Anterior end of suture between premaxilla and maxilla, 3. Anterior end of nasal, 4. Lateralmost point of nasal, 5. Mostanterior point of frontoparietal, 6. Lateralmost point of nasal (posterior), 7. Middle point of the median edge of frontoparietal, 8. Mostposterior median point of frontoparietal, 9. Posterior end of suture between frontoparietal and prootic, 10. Anterior end of suture between frontoparietal and prootic, 11. Middle point of the lateral edge of frontoparietal, 12. Mostanterior point of squamosal, 13. Posterior end of squamosal in contact with prootic, 14. Mostposterior point of prootic, 15. Mostposterior point of maxilla, 16. Posterior end of quadrate, 17. Medial tip of occipital. B. Ventral side: 1. Anteriormost point of premaxilla, 2. Anteriolateral end of premaxilla, 3. Mostposterior median end of premaxilla, 4. Posteriolateral end of premaxilla, 5. Lateralmost point of vomer, 6. Mostposterior end of vomer, 7. Most median point of palatine, 8. Anteriolateral end of palatine, 9. Posteriolateral end of palatine (anterior end of suture between palatine and pterygoid or anterior end of pterygoid), 10. Posteriomedian end of pterygoid, 11. Posteriolateral end of pterygoid, 12. Lateralmost end of parasphenoid, 13. Mostanterior median end of parasphenoid, 14. Mostposterior median end of parasphenoid, 15. Posterior end of maxilla in contact with quadratojugal, 16. Posterior end of quadratojugal, 17. Mostposterior end of occipital condyl.

Table 1. Means and standard errors (SE) of dorsal and ventral cranium sizes (CS) of male and female *Bufo bufo* and *B. verrucosissimus* (N: number of specimens).

Species	Sex	N	DC (CS _{means} ± SE)	N	VC (CS _{means} ± SE)
<i>B. bufo</i>	Males	10	57.379 ± 1.804	10	59.086 ± 1.052
	Females	10	79.713 ± 1.461	10	86.727 ± 2.068
<i>B. verrucosissimus</i>	Males	14	61.477 ± 1.823	14	66.289 ± 2.135
	Females	14	85.030 ± 3.119	14	91.461 ± 3.701

Table 2. ANOVA results for dorsal and ventral skull sizes in male and female *B. bufo* and *B. verrucosissimus*.

	DC				VC			
	df	MS	F	P	df	MS	F	P
Males								
Species	1	97.97	2.401	0.1355	1	302.61	7.165	0.0138
Populations nested within species	4	148.23	6.996	0.0012	4	239.98	16.772	<0.0001
Females								
Species	1	164.9	1.848	0.1877	1	130.7	0.999	0.3283
Populations nested within species	4	188.5	2.609	0.0682	4	333.1	3.775	0.0201

Table 3. MANOVA results for dorsal and ventral skull shapes in male and female *B. bufo* and *B. verrucosissimus* (df: degrees of freedom, F: F-test statistic, P: statistical significance).

	DC					VC				
	Wilks' lambda	df ₁	df ₂	F	P	Wilks' lambda	df ₁	df ₂	F	P
Males										
Species	0.000010	22	1	4387.947	0.0119	0.000385	22	1	118.0675	0.0724
Populations nested within species	0.000002	76	6.3296	2.29020	0.1393	0.000000	76	6.3296	3.35643	0.0578
Females										
Species	0.022553	22	1	1.970041	0.5163	0.000361	22	1	125.7074	0.0703
Populations nested within species	0.000000	76	6.3296	4.73118	0.0241	0.000000	76	6.3296	8.0505	0.0055

verrucosissimus were compared on the deformation grids, there were differences in the skull shapes of the species (Figure 4). *B. bufo* had larger squamous than *B. verrucosissimus*, whereas *B. verrucosissimus* had longer maxilla but shorter occipital region than *B. bufo* for both males and females.

When we controlled for size by using MANCOVA analysis (Table 4), we found species do not differ in skull shape both for males and for females. The MANCOVA analysis of shape revealed that there is no species and CS effect, and no significant CS x species interaction on dorsal and ventral skull shape (Table 4).

4. Discussion

The *Bufo bufo* species group distributing in western Palearctic consists of four species, named as *B. bufo*, *B. eichwaldi*, *B. spinosus* and *B. verrucosissimus*. Even though the presence of *B. bufo* and *B. verrucosissimus* in Turkey has been known, the range boundaries and taxonomic situations of these two species have been accepted uncertain up to now. Most recently, Özdemir et al. (2020) presented new morphological and molecular data clarifying to the identification and distribution of the Turkish *Bufo* species. According to the phylogenetic results, there are two main clades for specimens named as

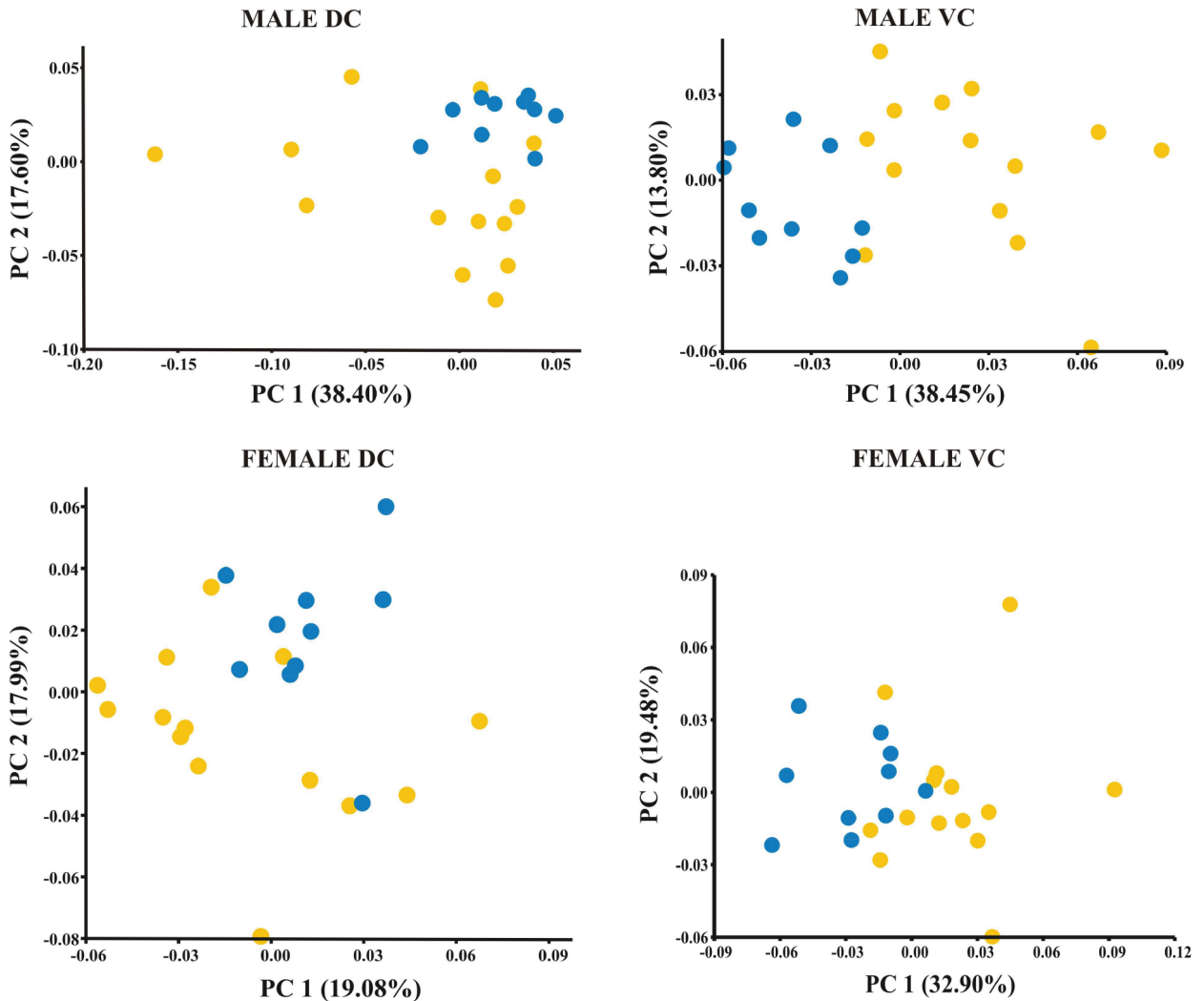


Figure 3. The positions of the specimens in morphospaces defined by the first two principal axes derived from covariance matrices of skull shape variables. Blue dots; *B. bufo*, yellow dots; *B. verrucosissimus*. DC: dorsal cranium, VC: ventral cranium.

B. bufo and *B. verrucosissimus* clade in Turkey. The latter clade also displayed a separation as two different subclades for Mediterranean specimens and northern specimens (Özdemir et al., 2020). For this reason, we chose our populations according to the molecular results of Özdemir et al. (2020) to see if there is any variation between *B. bufo* and *B. verrucosissimus*. We compared two populations of *B. bufo* and three populations of *B. verrucosissimus* in the present study. We examine a total of 48 skulls from both species.

According to our results, females in both species have larger skulls than males on both dorsal and ventral sides (Table 1). Evolutionary processes such as sexual and natural

selection are playing an active role on the morphological variability of males and females (Fairbairn, 1997; Hendry et al., 2014; Krstičić Račković et al., 2019). The difference between sexes, therefore, might be assumed a consequence of interactions of each sex with environment and between sexes. Krstičić Račković et al. (2019) reported for European brown frogs that females have larger ventral cranium and body size contrary to males, thus sexual dimorphism in cranial size is an indirect consequence of natural selection promoting larger body for higher fecundity in explosive breeders like brown frogs. Additionally, this hypothesis was reported for *B. bufo* species in different studies (Halliday and Verrel, 1986; Cvetković et al., 2007; Cadjenovic et al.

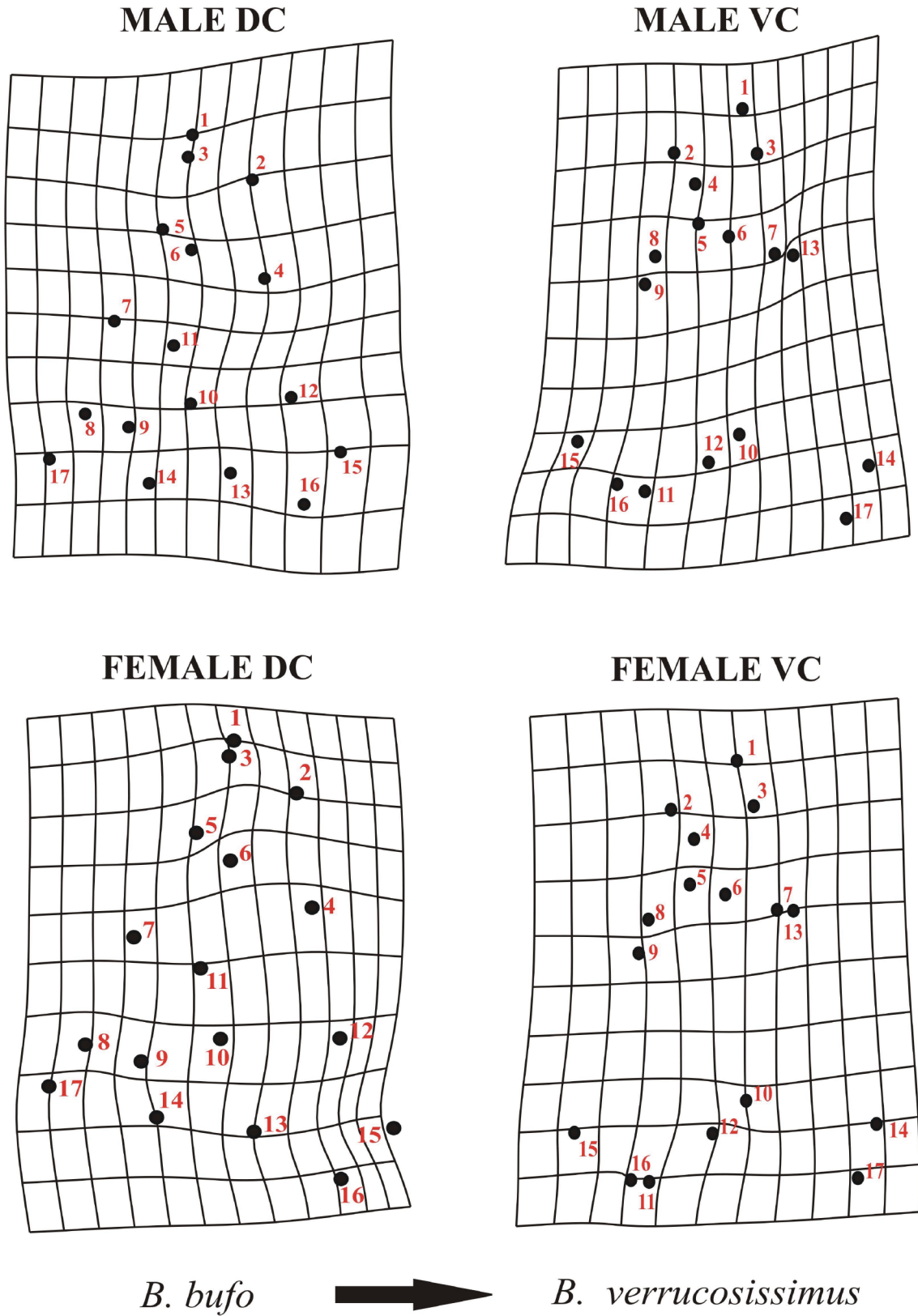


Figure 4. Cranium shape changes between *B. bufo* and *B. verrucosissimus*.

Table 4. The effects of species, size and their interaction for dorsal and ventral skull shape in the *Bufo* species group, tested by a multivariate analysis of covariance (MANCOVA).

	DC					VC				
	Wilks' lambda	df ₁	df ₂	F	P	Wilks' lambda	df ₁	df ₂	F	P
Males										
Species	0.003801	20	1	13.10289	0.2148	0.0009150	20	1	5.414232	0.3280
Size (CS)	0.007121	20	1	6.97131	0.2911	0.007059	20	1	7.032937	0.2899
CS x species	0.005697	20	1	8.72683	0.2615	0.008503	20	1	5.830492	0.3168
Females										
Species	0.213811	20	1	0.183851	0.9697	0.027009	20	1	1.801260	0.5351
Size (CS)	0.072345	20	1	0.641134	0.7739	0.045445	20	1	1.050236	0.6592
CS x species	0.232141	20	1	0.165386	0.9768	0.0024444	20	1	1.995520	0.5128

2013) Therefore, these selection pressures might be taken into consideration in terms of the variations between sexes that we observed.

Significant variation in skull size was found only for male ventral cranium between *B. bufo* and *B. verrucosissimus*. Size of dorsal skull in males and size of ventral skull in males and females significantly differed among populations within species (Table 2). Significant variation in skull shape were found only for male dorsal cranium between species, while both dorsal and ventral skulls show a significant variation among populations in females (Table 3). However, if we look the shape space which was defined by the first two principal components (PC1, PC2) obtained from dorsal and ventral skull shape variables indicate that *B. bufo* and *B. verrucosissimus* largely discriminated for both sexes although the dorsal skull of male individuals appears to be partially overlapped (Figure 3). Similarly, the Procrustes distances (PD) measured between both species for both dorsal and ventral craniums were found to be statistically significant as well. When we controlled for size, the MANCOVA analysis of shape revealed that there is no species and CS effect, and no significant CS x species interaction on dorsal and ventral skull shape (Table 4). Sanna (2019) studied on cranial morphology with two widespread European toad species *B. bufo* and *B. spinosus*. He reported similar allometric growth patterns characterized by cranial widening with relative shortening and dorsoventral compression. However, he found some interspecific shape differences characterized by a relatively shorter skull and a more dorsally extended snout distinguishing *B. spinosus* from *B. bufo*. In our samples, *B. bufo* had larger squamous than *B. verrucosissimus*, whereas *B. verrucosissimus* had longer maxilla but shorter occipital region than *B. bufo* for both males and females.

The similarity in the size and shape of skull mainly found in this study could result from structural and functional constraints because of living in similar environments or shared evolutionary history (Gould 2002; Blomberg et al. 2003; Losos 2011; Üzüüm et al., 2015). According to the molecular calibration of Recuero et al. (2012) and Garcia-Porta et al. (2012), the splitting time of *B. bufo* and *B. verrucosissimus* was reported around Late Pliocene and Early Pleistocene. In this period, the Caucasian Isthmus gradually collided with Anatolia and shaped the Lesser Caucasus Mountain Belt then subsequent glacial – interglacial cycles dominated the time until Holocene. This phylogeographic scenario is potentially describing the separation of both species. The presence of *B. verrucosissimus* in the Mediterranean Region can be associated with the recolonization events during the Pleistocene along Anatolian Diagonal reported in different species as well (Veith et al., 2003; Jandzik et al., 2013).

Also, *B. bufo* and *B. verrucosissimus* have the same habitat preferences: they inhabit in mainly forests, bushlands, and wet sites with dense vegetation or pebbly areas with sparse vegetation, and spend the daytime fossorially, sheltering under rocks or in soil. They have a similar diet; forage nocturnally, preying on insects, earthworms and some mollusks. Reproduction and spawning take place in lakes, ponds, ditches, large puddles and streams (Baran et al., 2012).² Climatic factors can indirectly affect skull morphology, as it determines the type and availability of food (Simon et al., 2016). Alternative climate-driven factors could also induce skull differentiation, such as reproduction sites (water pools) occurrence, and the ability of toads to detect them, which involves the olfactory capsules in the snout region (Trueb,

² AmphibiaWeb (2020). Website <http://amphibiaweb.org>. [accessed 29 July 2020].

1993; Simon et al., 2016; Sanna, 2019). So, all factors listed above could produce the observed similarities.

Although the morphological variations between *B. bufo* and *B. verrucosissimus* were described in the light of potential factors causing the similarities, genealogical relations, the other actor of the integrative taxonomy (Padial et al., 2010), should be taken into consideration. The *B. bufo* species group was subjected to various molecular based studies and the species were reported as the most recent sister species in the group (Recuero et al., 2012; Garcia-Porta et al., 2012; Arntzen et al., 2013; Özdemir et al., 2020). Gvoždik et al. (2008) implied that closely related species inherit most of their traits from their common ancestor, therefore they are phenotypically similar. This inference can be used to assess genotype – phenotype interaction. Hybridization, also, might be a reason finding out morphological similarities between species of which distribution ranges overlapping. This phenomenon comprehensively investigated between *B. bufo* and *B. spinosus* species in the border of France (Arntzen et al., 2016; 2017; 2018; 2020). Given that the initial splitting time of the *B. spinosus* from *B. bufo* is around 9 Mya following the orogenesis of Pyrenees and still able to hybridize, this

scenario can be valid in the Anatolia as well. Özdemir et al. (2020) pointed the presence of potential ongoing hybridization between *B. bufo* and *B. verrucosissimus* and the requirement of the population genetic studies to settle the issue. For this reason, this is a candidate view supporting the obtained results of this study.

Finally, in order to better reveal the skull variations between these two closely related species, it would be more appropriate to study more samples. Besides, it can be more clearly revealed whether the size and shape difference in the skull is related to gender.

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