Variation in skull size and shape of two snake species (Natrix natrix and Natrix tessellata)

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ORIGINAL ARTICLE

Variation in skull size and shape of two snake species (*Natrix natrix* and *Natrix tessellata*)

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Abstract We examined morphological differences in cranium size and shape between closely related snake species, *Natrix natrix* and *Natrix tessellata* (Natricinae, Colubroidea), as well as variation within species. These two snake species have similar ecology and habitat preferences but differ in feeding strategies. Our hypothesis was that divergence in size and shape of cranial elements between species depends on their functional role and anatomical relationships. To analyse complex, kinetic crania, we applied computed microtomography and 3D geometric morphometrics. We analysed size and shape of six cranial elements separately. We selected two "non-trophic" structures (akinetic braincase and mobile nasals) and four movable "trophic" skeletal elements (maxillae, quadrates, pterygoids and compound bones) which are involved in prey capture and swallowing. Our results showed that *N. natrix* and *N. tessellata* significantly differ in size and shape of all analysed cranial elements. In both species, cranium is significantly larger in females than in males. To account for possible differences in shape due to differences in size, we estimated allometric and non-allometric component of shape variation. For all elements, except nasals, allometry accounted for a significant proportion of the variance in shape. The analysis of non-allometric component of shape variation revealed significant dimorphism in shape of the braincase and maxilla between *N. tessellata* females and males, and marginally significant sexual dimorphism in shape of maxilla in *N. natrix*. These results indicated that sexual dimorphism in skull shape is species specific and not entirely caused by selection for larger size in females.

Keywords Natricinae · Geometric morphometrics · Cranium · 3D models · Sexual dimorphism · Allometry

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Introduction

The cranium or skull is the most complex skeletal structure comprising several highly integrated structural and functional units (Hanken and Hall 1993). Functionally, the skull is involved in feeding, protecting the central nervous system and housing sense organs, but also in locomotion and social interactions, mate competition, courtship and optionally for defence mechanisms (Smith 1993; Cundall 2000; Cundall and Greene 2000; Shine et al. 2000; Herrel et al. 2007).

In snakes, cranium is composed of numerous mobile and articulated elements and it is highly kinetic (Smith 1993; Herrel et al. 2007). The cranium of snakes consists of: (1) rigid braincase with anterior (parietals, frontals and parasphenoid) and posterior part (basisphenoid, basioccipital, supraoccipital, exoccipitals, opisthotics and prootics); (2) the snout complex (premaxillae, nasals, septomaxillae and vomers); (3) upper jaw (maxillae, palatines, pterygoids

and ectopterygoids); and (4) lower jaw (mandibles) consisting of dentaries, splenials, angulars and compound bones (Bellairs 1949; Rieppel 1980, 2007; Dwyer and Kaiser 1997; Polachowski and Werneburg 2013). Most snakes do not have a mandibular symphysis. Mandibles are interconnected by ligaments, while quadrates are movably articulated with the supratemporal bones. Such organisation of lower jaw and suspensorium allows independent movement of elements on the left and right side (Kardong 1977, 1979; Cundall and Gans 1979).

Skull features are indicators of diet in snakes, due to specialisations for catching, immobilising and ingesting prey (Gans 1961; Cundall and Greene 2000). In a group of advanced snakes (Colubroidea), swallowing is a conservative mechanism, while the prey capture and ingestion mechanisms are variable, displaying some differences in structure and cranial morphologies (Cundall 1983). The palato-pterygoid bars are used in prey ingestion transport by alternately ratcheting over the prey—"pterygoid walk" (Boltt and Ewer 1964). Movements of the mandible and palato-pterygoid bar are conservative, while the movement of the maxilla is variable (Cundall 1983). The mandible primarily keeps the prey against the roof of the mouth, while maxilla has a marginal role in swallowing (Kardong 1979, 1980), but a main role in prey capture (Smith 1993). The braincase is considered appropriate for taxonomic comparison in snakes (Kramer 1980; Gloyd and Conant 1990) and less influenced by selective pressures related to diet (Gentilli et al. 2009). Camilleri and Shine (1990) also defined braincase and nasals as "non-trophic" structures, while maxilla, compound bone, quadrate, pterygoid are defined as trophic structures. Therefore, it could be expected that divergence in skull elements between species depends on their functional and anatomical interrelationships.

In this study, we explored morphological variation in cranium size and shape of two sister species—the grass snake, *Natrix natrix* (Linnaeus, 1758), and the dice snake, *Natrix tessellata* (Laurenti, 1768). These two species diverged from the common ancestor 13–22 million years ago (Guicking et al. 2006). Both species have similar ecological preferences, but they differ in diet preferences: dice snakes feed predominantly on fish (Mebert 2011; Šukalo et al. 2014), whereas the grass snakes feed mostly on anurans (Luiselli and Rugiero 1991; Beebe and Griffiths 2000). These two species also differ in foraging strategies: the grass snake is an active forager, while the dice snake is a sit-and-wait predator or an active forager (Gruschwitz et al. 1999). They are non-venomous, and they do not constrict their prey (like most colubrids), but they swallow their prey alive (Borczyk 2015). *N. natrix* and *N. tessellata* use their heads primarily for feeding (not for male-to-male combat, courtship, burrowing or defensive biting).

We selected six cranial elements with different functional and anatomical relationships: "non-trophic" akinetic braincase (composed of several firmly connected bones) and movable nasals and four movable skeletal elements (maxilla, quadrate, pterygoid and compound bones) which are directly involved in prey capture and swallow actions. *N. natrix* and *N. tessellata* skull and position and relationship between skeletal elements are presented in Fig. 1 and Online Resources 1, 2.

Our main goal was to explore the morphological divergence in cranium size and shape between *N. natrix* and *N. tessellata* as well as variation within species. We aimed to answer the following questions: Do species diverge in skull size and shape? Do the braincase and movable bones show a similar pattern of interspecific and intersexual differences? Are the differences in shape caused by allometric, size-related, shape changes?

Materials and methods

Analysed localities and samples

In this study, we used 66 alcohol-preserved adult specimens of *N. natrix* ($n = 25$) and *N. tessellata* ($n = 41$), collected at the localities of Pančevački Rit (Serbia, vicinity of Belgrade), Obedska bara (Serbia, vicinity of Belgrade) and Golem Grad Island (FYR of Macedonia, Prespa Lake). See Supplementary Table 1 for localities and sample information. Specimens used for this study were either initially collected for ecotoxicological studies (approved by the Serbian Ministry for Energy, Development and Environmental Protection, Permissions Nos: 353-01-640/2012-03 and 353-01-77/2013-08) or killed (trampled on roads, drowned in fishing nets). Sex and reproductive maturity were determined by the morphology of the gonads.

Computed tomography, landmark scoring and shape variables

Specimens were scanned with a computed microtomograph Skyscan 1172 100 kV under following settings: aluminium filter 0.5 mm, 74 kV, 0.8 rotation step, 515 ms exposure time, four frames average. 3D surface models of snake skulls were produced using Skyscan CT-analyser, version 1.10 software, with a resolution of 26.1 μm. 3D landmark coordinates were recorded from the surface models using Landmark IDAVv3.0 (http://graphics.idav.ucdavis.edu/research/EvoMorph). The braincase and movable skeletal elements were digitised separately. Landmarks were scored on both sides of the skull: 32 landmarks on the braincase, 8 on nasals, 8 on pterygoid, 14 on maxilla, 6 on quadrate and

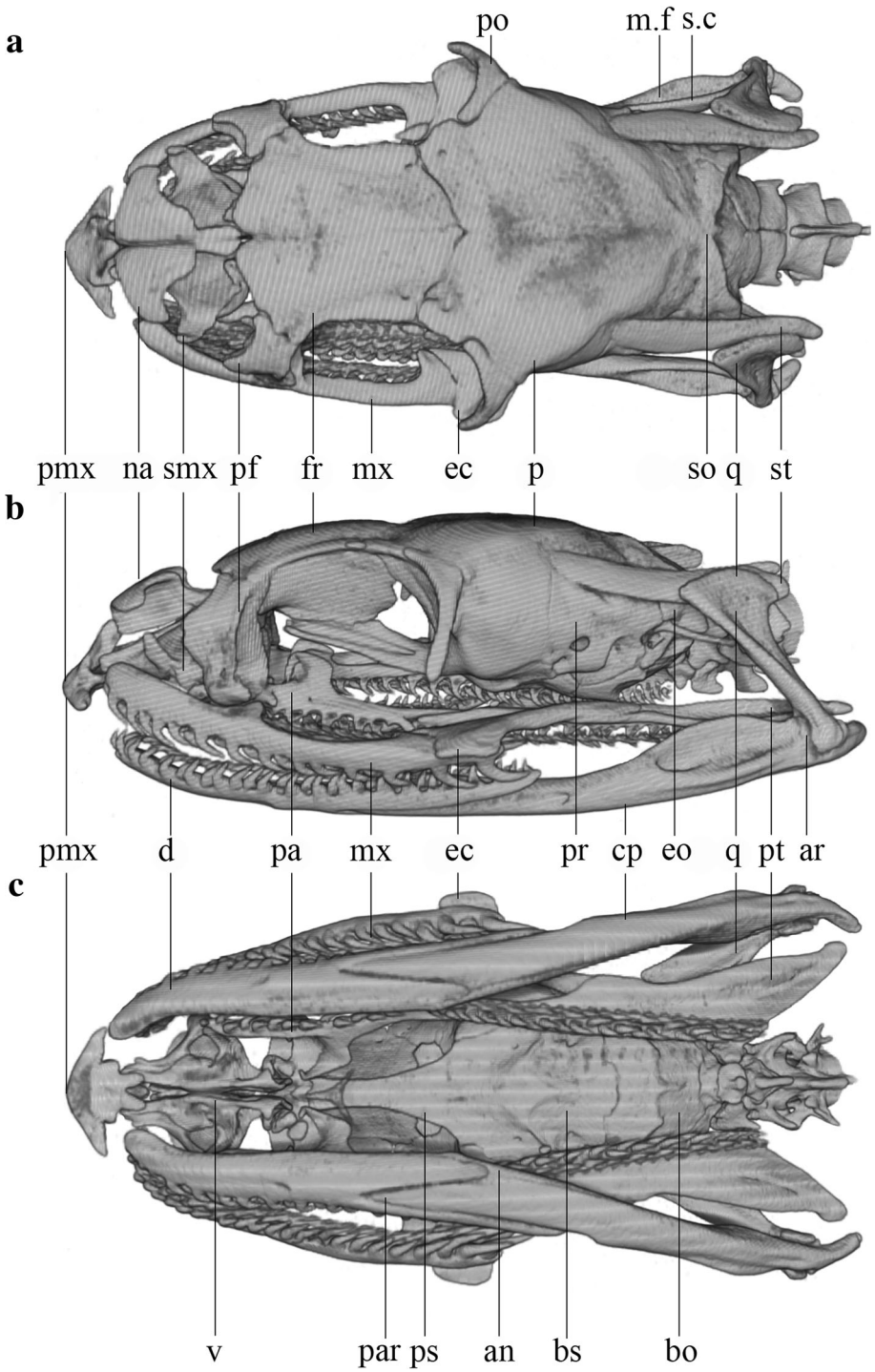


Fig. 1 3D model of the *N. natrix* skull in dorsal (**a**), lateral (**b**) and ventral (**c**) projection. *an* angular, *ar* articular, *bo* basioccipital, *bs* basisphenoid, *cp* compound bone, *d* dentary, *ec* ectopterygoid, *eo* exoccipital, *fr* frontal, *m. f* mandibular fossa, *mx* maxilla, *na* nasal, *p* parietal, *pa* palatine, *par* prearticular, *pf* prefrontal, *pmx* premaxilla, *po* postorbital, *pr* prootic, *ps* parasphenoid, *pt* pterygoid, *q* quadrate, *s. c* surangular crest, *smx* septomaxilla, *so* supraoccipital, *st* supratemporal, *v* vomer

11 on compound bone (detailed description of landmarks is provided in Supplementary Table 2).

Centroid size (CS), the square root of the summed squared distances between all landmarks and their centroid, was calculated for each skeletal element separately. Shape variables were obtained by Procrustes superimposition (Dryden and Mardia 1998). For skeletal elements with object symmetry (braincase and nasals), the Procrustes fit accounted for object symmetry was performed and the shape variables for the symmetric component of shape were extracted (Klingenberg et al. 2002). For paired structures with matching symmetry (pterygoids, maxillae, quadrates and compound bones), we used average values of a cranial element on the left and the right side of the skull.

Statistical analyses

The differences in size (CS) between the species and sexes were tested by analysis of variance (ANOVA). A principal component analysis (PCA) was applied to explore variation and visualise divergence in shape between *N. natrix* and *N. tessellata* and variation in shape within each of analysed species. For braincase and nasals, covariance matrices of symmetric component of variation were calculated. For other skeletal elements with matching symmetry, covariance matrices of Procrustes coordinates were calculated and subsequently used for PCA.

Allometry was estimated with a multivariate regression of shape variables on CS. The amount of size-dependent shape variance was quantified as the percentage of total shape variance explained by size, and its statistical significance was estimated using a permutation test with 10,000 iterations against the null hypothesis of independence between size and shape (Good 1994; Klingenberg 2011). Residuals from the multivariate regression of shape variables on CS were used in further analyses to explore the non-allometric component of shape variation.

To test divergence in shape of skeletal elements, we calculated Procrustes distance (PD)—the square root of the sum of squared distances between pairs of corresponding landmarks. PD between mean shapes of skeletal elements of females and males was calculated twice—for allometry-included and allometry-corrected component of shape variation. The statistical significance of PD was obtained by a permutation test with 10,000 iterations.

Procrustes superimposition, PCA, multivariate regression and visualisation of shape changes were carried out using MorphoJ software (Klingenberg 2011), while ANOVA was performed in R (R Development Core Team 2012). Visualisations of shape changes were done using Landmark IDAVv3.0 and MeshLab_64bit v1.3.3 software (http://meshlab.sourceforge.net/).

Table 1 The difference in size of skeletal elements between species (ANOVA test)

	Females			Males		
	SS	$F_{(1, 35)}$	p	SS	$F_{(1, 27)}$	p
Braincase	0.000073	6.07	0.019	0.000036	5.51	0.027
Nasal	0.000003	5.42	0.026	0.000001	3.97	0.057
Pterygoid	0.000144	17.99	0.001	0.000096	26.54	0.001
Maxilla	0.000284	59.24	0.001	0.000130	38.41	0.001
Quadrate	0.000056	16.81	0.001	0.000031	19.56	0.001
Compound	0.000073	6.54	0.015	0.000071	14.14	0.001

SS sum of squares, *F* F test statistic, degrees of freedom (model df, error df), *p* statistical significance

Fig. 2 The position of the individuals in the morphospace defined by the first two PC axes and visual presentation of the individuals with the minimal and the maximal values of PC1 scores for any particular element. The skeletal elements are *shaded*. For the presentation of pterygoid shape, a part of the lower jaw was digitally removed. *Black dots—N. natrix*, *empty dots—N. tessellata*

Results

Divergence in size and shape between *N. natrix* and *N. tessellata*

Mean values and standard errors of CS for six cranial elements in females and males of *N. natrix* and *N. tessellata* are presented in Supplementary Table 3. Mean values of CS were significantly bigger in *N. tessellata* than in *N. natrix* for all analysed elements (Table 1).

The positions of the individuals in the morphospace described by the first two PC axes are shown in Fig. 2. Species are clearly separated by the first PC axis which describes the largest percentage of variance in shape for each of the analysed cranial elements. The shape variation within the species was depicted by PC2.

Braincase (Fig. 2a): *N. natrix* has sturdier braincase than *N. tessellata*. The braincase is much wider in *N. natrix* with a relatively elongated and wider frontals, wider and larger parietals, larger basisphenoid and parasphenoid than *N. tessellata.* This species has wider, shorter and more anteriorly positioned occipital region (supraoccipital and basioccipital) than *N. tessellata.*

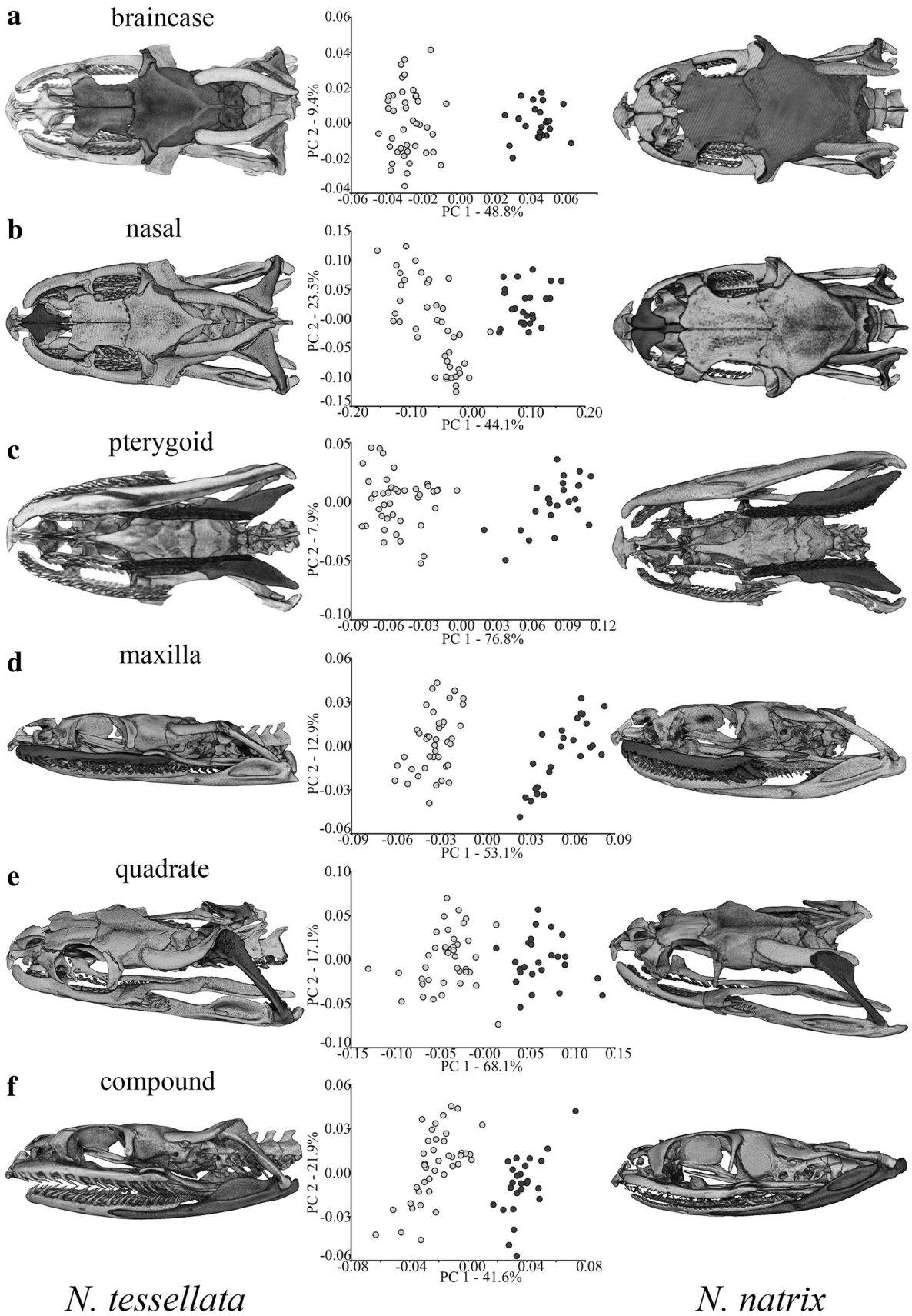
Nasals (Fig. 2b): Species clearly diverge in shape of nasals, which are much wider and sturdier in *N. natrix.*

Pterygoid (Fig. 2c): *N. natrix* has a relatively shorter anterior part and wider posterior part, larger (longer and wider) and anteriorly positioned ectopterygoid process than *N. tessellata.*

Maxilla (Fig. 2d): *N. natrix* has a relatively shorter and wider maxilla, smaller and anteriorly positioned ectopterygoid process, smaller and posteriorly positioned palatine process and more elongated posterior part than *N. tessellata.* In *N. natrix,* maxilla is curved and bears large teeth on its posterior part.

Quadrate (Fig. 2e): *N. natrix* has relatively shorter quadrate, elongated and wider dorsal part (articulation surface with supratemporal) and smaller articulation surface with lower jaw than *N. tessellata*.

Compound (Fig. 2f): Compared to *N. tessellata*, *N. natrix* has shorter compound bone, narrower surangular crest and mandibular fossa and shorter articulation surface with quadrate.


a
braincase
PC 2 - 9.4%
PC 1 - 48.8%
b
nasal
PC 2 - 23.5%
PC 1 - 44.1%
c
pterygoid
PC 2 - 7.9%
PC 1 - 76.8%
d
maxilla
PC 2 - 12.9%
PC 1 - 53.1%
e
quadrate
PC 2 - 17.1%
PC 1 - 68.1%
f
compound
PC 2 - 21.9%
PC 1 - 41.6%
N. tessellata
N. natrix

Variation in size and shape of cranial elements within species

There is a significant sexual dimorphism in size for all analysed skeletal elements in both species (Table 2). The cranial elements are significantly larger in females, being 16–39 % larger than in males (Table 2). Females and males significantly diverge in shape of braincase, maxilla and nasals in *N. natrix*, while females and males of *N. tessellata* diverge in shape of braincase, maxilla and compound bone (Table 2). Multivariate regressions of shape on the CS were statistically significant for braincase, pterygoid, maxilla and compound bone in *N. natrix* and for braincase, pterygoid, maxilla, quadrate and compound bone in *N. tessellata* (Figs. 3, 4, respectively). The results of multivariate regression analyses revealed that relatively large portion of variance in shape is caused by allometric, size-related shape changes (from 10.9 to 22.6 %, see Figs. 3, 4).

Braincase (Figs. 3a, 4a): Larger individuals have a wider braincase than smaller ones in both *Natrix* species. In *N. natrix* (Fig. 3a), the allometric shape changes were related to the change in shape of the anterior part of frontals, posterior part of parietals, supraoccipital and exoccipitals. These changes were visible on the dorsal projection of the combined model (Fig. 3a). In *N. natrix*, the shape changes of the posterior part of basioccipital, parasphenoid and medial part of parietals (visible on the ventral projection of the combined model) are the changes in shape associated with changes in size. Larger individuals of *N. natrix* have also relatively narrower postorbitals. In *N. tessellata,* allometric shape changes of braincase are similar to the shape changes in *N. natrix* (Fig. 4a). These shape changes are related to the relative change in shape of frontals, anterior and medial part of parietals, medial part of supraoccipital and exoccipitals (visible on dorsal projection of combined model), posterior part of basioccipital and change in the relative position of postforantals which are more posteriorly positioned in larger individuals.

Pterygoid: In *N. natrix* (Fig. 3b), larger individuals have more elongated and wider pterygoids, especially the anterior, teeth-bearing part. In *N. tessellata* (Fig. 4b), larger individuals have wider and higher pterygoids.

Maxilla: In *N. natrix* (Fig. 3c), anterior part of maxilla is relatively wider and more elongated in larger individuals and their palatal and ectopterygoid processes are closer to one another than in smaller ones. In *N. tessellata* (Fig. 4c), maxilla is more elongated and wider at the level of process, and the palatal and ectopterygoid processes are wider and closer to one another in larger individuals.

Quadrate: In *N. natrix* (Fig. 3d), allometry is not statistically significant, while in *N. tessellata,* larger individuals have a proportionally narrower quadrate (especially the articulation surface with supratemporal) than smaller ones (Fig. 4d).

Compound: In *N. natrix,* larger individuals have relatively larger mandibular fossa and articulation surface with quadrate than smaller ones (Fig. 3e). In *N. tessellata,* compound bones were shorter and wider in larger individuals with larger and more posteriorly positioned articulation surface with quadrate than in smaller individuals (Fig. 4e).

When non-allometric component of shape variation (residuals obtained from multivariate regression) was analysed, significant differences between females and males were found for shape of maxilla in *N. natrix* (PD = 0.023, $p = 0.047$), and for braincase (PD = 0.017, $p = 0.007$) and maxilla (PD = 0.021, $p = 0.001$) in *N. tessellata*.

Discussion

Natrix natrix and *N. tessellata* significantly differ in skull size and shape. *N. natrix* have wider, sturdier cranium, while *N. tessellata* have a more elongated and narrower cranium. Observed differences in size and shape between the species could be related to the differences in their diet, prey size, foraging strategies, feeding performances and handling abilities (Shine 1991; Forsman and Shine 1997; Adams and Rohlf 2000; Krause et al. 2003; Herrel et al. 2008; Mori and Vincent 2008).

Table 2 Sexual dimorphism in size expressed as index value CS F/M (mean CS for females/mean CS for males and the statistical significance of sexual dimorphism in size obtained by ANOVA) and sexual dimorphism in shape PD F/M expressed in units of Procrustes distance and statistical significance of differences in shape obtained by permutation test in *N. natrix* and *N. tessellata*

	N. natrix				*N. tessellata*			
	Size		Shape		Size		Shape	
	CS F/M	*p*	PD F/M	*p*	CS F/M	*p*	PD F/M	*p*
Braincase	1.157	0.001	0.026	0.001	1.158	0.001	0.023	0.001
Nasal	1.162	0.001	0.058	0.028	1.186	0.001	0.017	0.721
Pterygoid	1.382	0.001	0.018	0.393	1.290	0.001	0.035	0.001
Maxilla	1.172	0.001	0.047	0.001	1.182	0.001	0.028	0.001
Quadrate	1.465	0.001	0.024	0.237	1.360	0.001	0.024	0.055
Compound	1.390	0.001	0.028	0.009	1.287	0.001	0.014	0.269

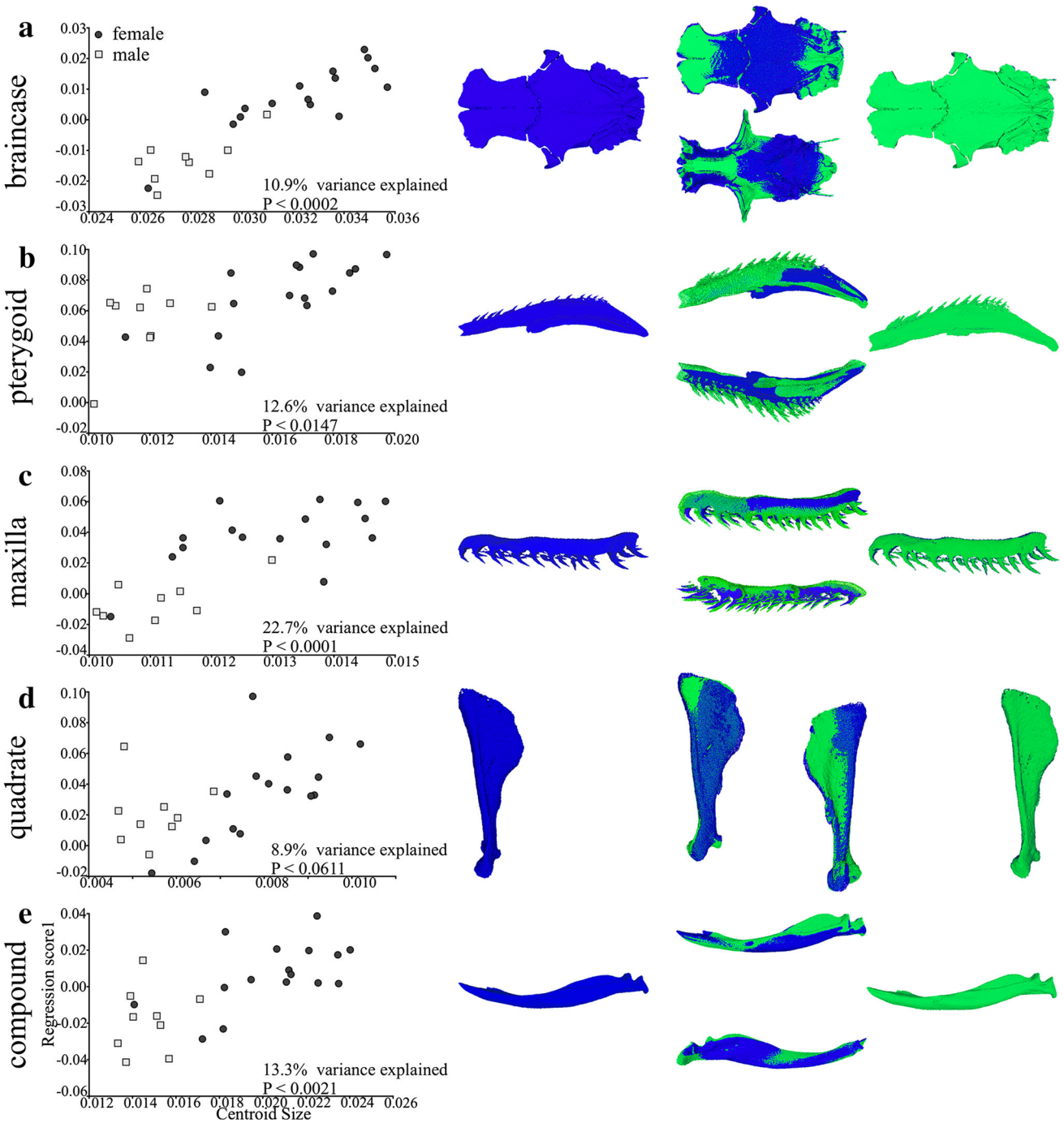


Fig. 3 Multivariate regression of shape variable on CS in *N. natrix*. The *dark grey* (*blue* online) models present a shape of the specimen with the minimal CS, and *light grey* (*green* online) models represent a shape of the specimen with the maximal CS. Models with *mixed colours* present combined models showing changes in shape related to the change in size. Combined models are presented with two pictures (*upper* and *lower*); the pictures of combined model show the dorsal and ventral projection of the braincase and the *left* pterygoid, and the lateral and the medial projection of the *left* quadrate and the *left* compound bone

In general, variation in relative head dimensions within and among snake taxa has effect on swallowing performance (Pough and Groves 1983; Forsman and Lindell 1993; King 2002).

The shape of the cranium in *N. natrix,* a frog-eating snake, could be the result of the functional and structural demands related to the stronger bite force. Increase in muscle force and bite force imposes greater loads on cranial elements which need to withstand them (Herrel et al. 2007; Mori and Vincent 2008; Borczyk 2015). On the other hand, more elongated and narrower cranium of *N. tessellata* with relatively longer jaws and larger gape may be

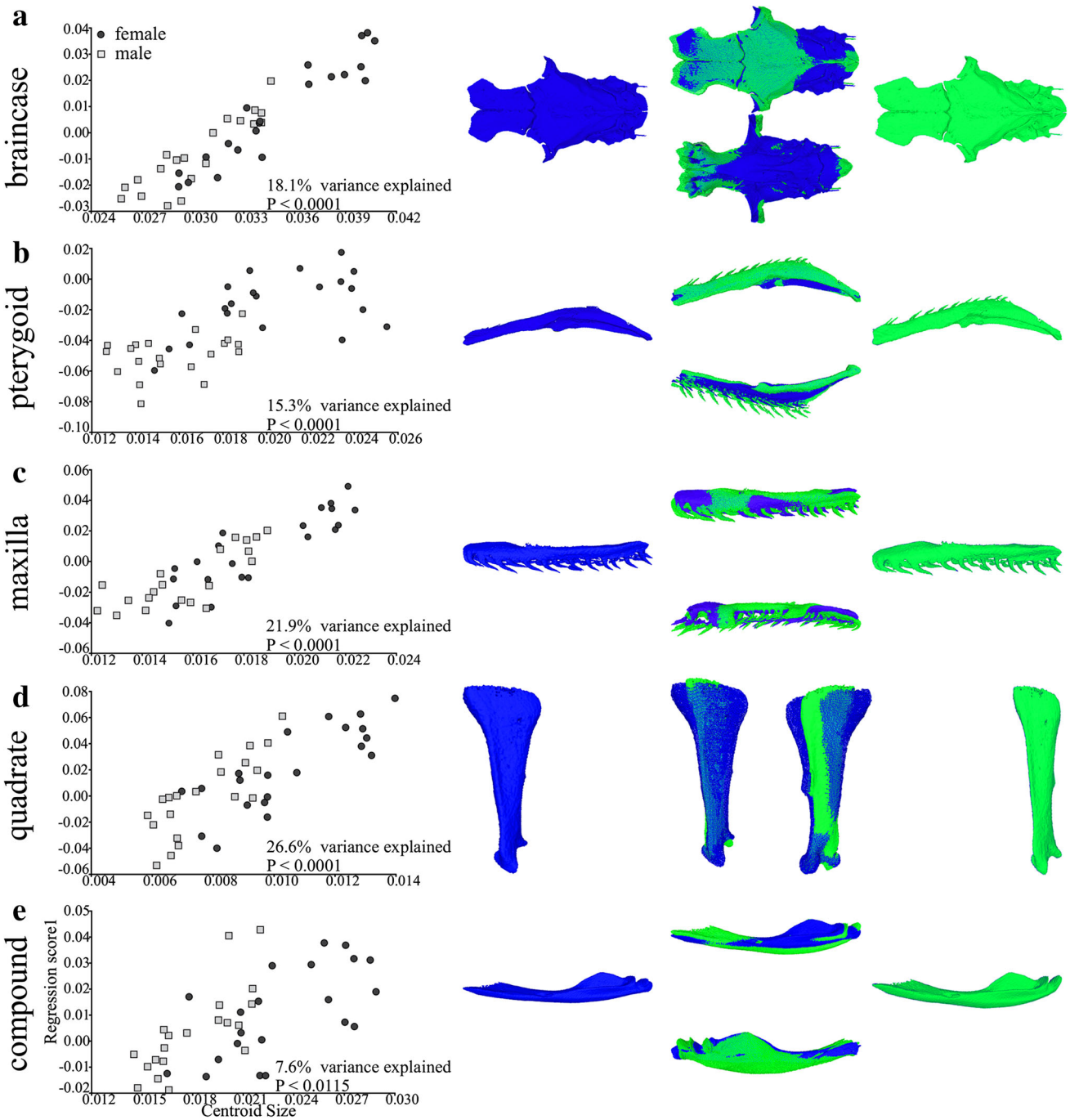


Fig. 4 Multivariate regression of shape variable on CS in *N. tessellata*. The *dark grey* (*blue* online) models present a shape of the specimen with the minimal CS, and *light grey* (*green* online) models represent a shape of the specimen with the maximal CS. Models with *mixed colours* present combined models showing changes in shape related to the change in size. The pictures of combined model show the dorsal and ventral projection of the braincase and the *left* pterygoid, and the lateral and the medial projection of the *left* quadrate and the *left* compound bone

beneficial for piscivory (Hampton 2011) and can minimise the costs of prey ingestion (Pough and Groves 1983; Forsman and Lindell 1993). The more hydrodynamical head shape of *N. tessellata* could also be regarded as an adaptation for faster forward striking speeds underwater (Hibbitts and Fitzgerald 2005; Herrel et al. 2008).

Our analyses indicate that the divergence in shape of movable cranial elements is more complex. In *N. natrix,* the majority of movable cranial elements (pterygoid and maxilla) are more robust, wider and shorter than in *N. tessellata*. *N. natrix* have longer muscle attachment surface on pterygoids, which could be correlated with stronger

handling during "pterygoid walk" (Boltt and Ewer 1964) in frog-eating species. Wider and shorter (more robust) maxilla of *N. natrix* could also be related to prey capture and ingestion of frogs. Also, the posterior part of maxilla is elongated in *N. natrix* and bears bigger posterior teeth comparing to *N. tessellata*. In *N. natrix,* the quadrate has a wider articulation with supratemporal, but narrower articulation with the lower jaw. When the compound bone is concerned, *N. natrix* has a narrower surangular crest and mandibular fossa. *N. tessellata* has relatively longer quadrate bones. It was found that increase in relative quadrate length decreases the swallowing time in fish-eating species (Vincent et al. 2009).

We also found substantial variation in size and shape of skeletal elements within species (Table 2). Allometry explains the significant percentage of the observed shape variation within the species. The allometric shape changes largely coincide with the observed between-sexes differences of movable, trophically related skeletal elements (pterygoids, quadrates and compound bones). However, sexes diverged in shape when allometry-free data were analysed. Such divergences in shape were found for braincase and maxilla in *N. tessellata* and for maxilla in *N. natrix.* Sexual dimorphism is a common phenomenon in snakes (Mertens 1947; Fitch 1981; King 1989; Arnold 1993; Shine 1994; Krause et al. 2003). In both analysed species, sexual dimorphism in size is well known and females tend to be the larger sex (Madsen 1983; Mebert 2011). Generally, sexual body dimorphism is driven by sexual selection (Darwin 1971), fecundity selection (Olsson et al. 2002; Cox et al. 2003) or ecological selection (Slatkin 1984; Shine 1989). Sexual dimorphism in the head size and shape of snakes is not sexually selected, as most species of snakes do not use the head or jaws in any kind of intraspecific combat, courtship or social interactions (Camilleri and Shine 1990; Shine 1991). It has been suggested that morphological divergence in body size and head shape in snakes could be coupled with divergence in feeding behaviour, diet and differential prey selection between the sexes (Schoener 1977; Shine 1986, 1993; Vincent and Herrel 2007). In *N. natrix,* dietary differences are accompanied by relatively bigger heads in females (Thorpe 1979; Kabisch 1999; Gregory and Isaac 2004). Hence, it can be expected that larger head (cranium) is an adaptation for acquiring food more efficiently.

In conclusion, *N. natrix* and *N. tessellata* have clearly marked difference in shape of the cranium, regardless of the functional role or anatomical relations among skeletal parts. Allometry explained the significant portion of the variance in shape within species. The differences between the sexes largely coincide with allometric shape changes. However, some differences in the pattern of variation between braincase and movable bones exist. Studies of integration and modularity of structural and functional units can provide better insight into the morphological changes and evolution of the cranial skeleton in snakes.

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