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Assessing geographic differences in skulls of *Neomys fodiens* and *Neomys anomalus* using linear measurements, geometric morphometrics, and non-metric epigenetics

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Abstract

Previous morphological studies on *Neomys fodiens* and *Neomys anomalus* describe a pronounced ecological variance, mainly attributed to altitudinal and/or climatic conditions especially for *Neomys fodiens*. The major aim of this study was to find out whether there are intraspecific geographic variations related to cranial morphometry. Two different methods were used: classical linear measurements and modern geometric morphometric 2D method. Shrew skulls from Germany and Slovakia separated into different regional groups were studied. For *Neomys fodiens*, the linear method showed a clearer separation than the geometric method, whereby the skull measures *CBL* and *CORH* followed Bergmann's rule, which could be explained with an allopatric living. Both methods produced various results for the characters in which the groups differed the most. For *N. anomalus*, the selectivity was high in both methods, with similar results. The linear skull measures were heterogeneous, which may possibly have been caused by an interspecific competition with *N. fodiens*. The lengths of the unicuspid teeth of the maxilla showed the strongest variation between the regions, which might be associated with a different prey selection. Likewise, a non-metric study on *N. fodiens* was performed to obtain knowledge about the epigenetic variability. There was no sign for significant epigenetic impoverishment ($I_{ev} = 0.42$), and the degrees of the epigenetic distances (MMD = 0.01 to 0.06) indicated a small differentiation between the *N. fodiens* groups. The fluctuating asymmetry (FA = 0.15 to 0.21) is rather small by comparison with other mammals. So, there is only a small indication of reduced developmental stability in all regional groups, but with an increase from south to north.

Keywords *Neomys* · Geometric morphometry · Linear morphometry · Epigenetics

Introduction

The variability of the structure and the shape of organisms are usually captured by employing morphometry (Reyment

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2010). This quantitative method enables the characterization of biological shapes, and its use reaches from a mere descriptive data acquisition for taxonomic classification to an analysis of the influence of ecological factors or mutations. Traditionally, standardized measurement data are collected which contain information about the length and width of structures or bones and the ratio or angles between them—thus, mainly giving size information (Polly 2012) or rather is a quantification of both size and shape of the object at the same time. A newer, digital approach is geometric morphometry (GMM), which analyzes biological shape using geometric coordinates ("landmarks") (Bookstein 1991). So, GMM is a quantification of shape only, even though it may be correlated with size.

Water shrews, the Eurasian water shrew *Neomys fodiens* (PENNANT, 1771), and the Mediterranean water shrew *Neomys anomalus* CABRERA, 1907, occur in the Palearctic region and have a wide distribution range (Kraft 2008; Spitzenberger 1990a, b) in Central Europe but are fairly

elusive. We noticed the latest molecular studies from Igea et al. (2015) which revealed a clear genetic differentiation between *N. anomalus* und *N. milleri* and elevated them as distinct species. But as the taxonomy was not in the focus of our study and for easy finding, we left the "old" names. Both species are listed as least concern by the IUCN and are not included in the appendices of the European Habitat Directive. Nevertheless, *N. fodiens* is on the early warning list of the German Red List and *N. anomalus* is a category "2" (strongly endangered, Meinig et al. 2009). Both are included in the BundesArtenschutzGesetz as "especially protected" and, therefore, the capturing possibilities are restricted. Respectively, investigating the morphology of these species is difficult due to fairly small samples at least in German collections.

So far, only few morphological studies on water shrews are available. A phenotypic plasticity pronounced in *N. fodiens* and less so in *N. anomalus* has been described and is most likely correlated to different altitudes (Spitzenberger 1980, 1990a; Ochocińska and Taylor 2003). There are also geographic variances from south to north and west to east, respectively (Spitzenberger 1990a, b). Some studies revealed different tendencies for the results in allopatry or sympatry (Rychlik et al. 2006) and another ecological difference for water shrews appeared in context with the precipitation rate and moisture grade of biotopes (Price 1953).

The aim of the study was to evaluate intraspecific geographic variations related to the skull morphology and morphometry using available but small samples from collections. Three different methods were employed: linear morphometric measurements, geometric morphometry, and a non-metric, epigenetic study. This approach also allows to assess the discriminating power of these methods in relation to the small samples. Another aim was to advance knowledge about the epigenetic variability in *Neomys fodiens* as this would constitute the first report of non-metric skull characters for this species.

Material and methods

We investigated a total of 228 skulls of *Neomys fodiens* and 65 skulls of *Neomys anomalus* from the collections of the Senckenberg Museum Dresden, Frankfurt, and Görlitz as well as from the Staatlichen Museum für Naturkunde Stuttgart and the Museum für Naturkunde Berlin.

The material of both species was classified by means of their locality into different regional groups (Fig. 1). For *N. fodiens*, it results in five groups and for *N. anomalus* in four groups (for the latter, a fifth regional group would have been possible, but it influenced the model accuracy in the linear analysis extremely negative because of many missing measurements and for the geometric method, the sample size was very small. So, we decided to waive this group). For each location, latitude and longitude were determined by using the website www.latlong.com and the height above sea level were searched to the nearest estimate (Online Resource 1–4). Altitudes ranged from about 29 to 1010 m above sea level, latitude from 47.49 to 52.57, and longitude from 8.67 to 20. The specimens were collected in different years and during different seasons.

For the evaluation of the potential dependence of the metric and non-metric character expression on age, the skulls were classified into four age groups (AGs) based on the wear of the red tooth tips (in accordance with Popov and Zidarova (2008)): AG 1 = none till low wear of the red tooth tips, 2 = recognizable wear of the red tooth tips, 3 = nearly total wear of the red tooth tips, 4 = total wear of the red tooth tips. The first two groups suggest juvenile or young adults, while group 3 and 4 correspond to sexually mature, already overwintered shrews (Churchfield 1990).

Linear morphometrics and statistics applied

For this analysis, 21 linear cranial measurements (Fig. 2) were taken with a digital caliber (150–0.01 mm). The data were subjected to descriptive statistics and the Kolmogorov-Smirnov test showed a normal distribution (Online Resource 5–7). The metric data were checked for homogeneity in age using the Pearson correlation coefficient ($p < 0.05$) and in sex using T test ($p < 0.05$) and Mann-Whitney U test ($p < 0.05$), when the variances between tested groups differed. A discriminant analysis (DA) was applied to detect intraspecific variation. The DA was done using Wilk's lambda statistic, entry of all variables at once not stepwise, with equal prior probabilities of group membership, based on the pooled within-group covariance matrix.

In order to see if there were correlations with altitude, longitude, and latitude, we used the Pearson correlation coefficient ($p < 0.05$). SPSS 21 was used for all the calculations.

The Pearson correlation coefficient showed significances between the age class and linear measurements (Online Resource 8). To eliminate the influence of these significances, shrews with age group 3 and 4 and the significant measure distances *Lgim3* and *Li* were excluded in both species. Leaving the sample size as given in Table 1. This is in accordance with Zidarova and Popov (2018) who also used only subadult specimens in their study of shrews including *Neomys*.

The results of the T test and the Mann-Whitney U test showed no significant differences between the sexes and the morphological characters for both species (Online Resource 9–11). So, the sexes were pooled for further study.

Fig. 1 Geographic locations of the shrew samples studied in Germany and Slovakia. Black dots—*N. anomalus*; open dots—*N. fodiens*. BW Baden Württemberg; GP Garmisch Partenkirchen, Bavaria; HEP Heppenheim, Hesse; HT High Tatras; MS Märkische Schweiz, Brandenburg; OLHT Upper Lausitz Heath- and Pond landscape, Saxony; PSA Plauen, Saxony; SOL Southern Upper Lausitz, Saxony

Geometric morphometrics

Checking both species for homogeneity in age and sex with an ANOVA revealed no significant age or sex correlation (Online Resource 12–15).

Also, only immature shrews were included to minimize the effect of age differences. Table 2 gives an overview of the final sample size. The shapes of the specimens were measured with two-dimensional landmarks. Each skull was photographed in ventral and lateral view, the mandibles in lateral view. Ten landmarks were set on the ventral view of the skull, 11 on the lateral view, and 10 on the mandible (Fig. 3). The landmarks were digitized with the tpsDig program (http://life.bio.sunysb.edu/ee/rohlf/software.html). To convert landmarks into shape coordinates, each dataset was Procrustes superimposed. So, the shapes were aligned and any differences in size, rotation, and translation were removed (Rohlf and Slice 1990). Also, a covariance matrix was generated. The centroid size is calculated based on distances between landmarks in their original units and was calculated for each specimen as the square root of the sum of the squared distances between the landmarks and the centroid (Dryden and Mardia 1998). The centroid size is based on the collective measure of all landmarks.

To assess variation in shape, a PC analysis was made. This variation was characterized on the basis of the first principal component (PC 1), the one that continues through the centroid of the longest extent of the cloud diagram and explains most of the variation in that dimension (Brosius 2006). The variation for all three skeletal views was illustrated using a thin-plate spline deformation.

A discriminant analysis with cross-validation was used to indicate whether regional groups can be distinguished reliably and which shape traits separate them the most. The results imported the Procrustes distance as the square root of the sum of squared corresponding landmarks of two superimposed shapes (Dryden and Mardia 1998). Thus, it is an index for shape differences. The degree of separation was calculated by the Mahalanobis distance (Zelditch et al. 2012):

$$D = \sqrt{(X_1 - X_2)^T S^{-1} (X_1 - X_2)}$$

where X_1 and X_2 = group centroid and S^{-1} = inverse covariance matrix.

The DA included a parametric T^2 test ($p \leq 0.05$) for the difference between group means. All the analyses were calculated with the MorphoJ 1.06d program (http://www.flywings.org.uk/morphoj_page.htm).

Epigenetic study

Epigenetic research aims mainly at detecting isolation problems of endangered species and an associated impoverished gene flow (Ansorge 2001). As semiaquatic mammals, dependence on moist biotopes is very distinctive for water shrews, and the various anthropogenic interventions constitute a potential threat (Kapischke 2009). Non-metric characters such as diverse kinds of discontinuous variations can be found in different skeletal parts (Ansorge et al. 2012). Mainly, skulls are

Fig. 2 Measurements used in linear analysis of *Neomys fodiens* and *Neomys anomalus* (drawings modified according to Spitzenberger ([1990b](#))). Abbreviation of measurements taken in alphabetical order: *CBL* condylobasal length; *CORH* coronoid height (distance between incisura praeangularis and top of processus coronoideus); *GSL* maximum skull length; *HM* mandibula height behind the coronoid; *HS* maximum skull height (from condyles to top of the skull, preferably at right angles to the skull base); *HSB* maximum skull witdh; *IOS* interorbital width; *Lg A1-A4* length of A1-A4; *Lg i-m3* length of i-m3; *Lg I-M3* length of I-M3; *Lg p-m* length of p3-m3; *Lg P4-M4* length of P4-M3; *Li* length of I (labial); *Lpo* from anterior edge of the maxilla to distal end of os palatinum; *LMA* length of mandibula from posterior end of incisivus to processus angularis; *LMC* maximum length of mandibula from posterior end of incisuvus to processus condylaris; *LPO* cranium length,from posterior end of os palatinum to posterior edge of foramen magnum; *LPS* from distal end of os palatinum to anterior edge of foramen magnum; *p.Pg* postglenoid width; *RB* rostrum width at the level of the first and second unicuspid; *ZW* zygomatic width
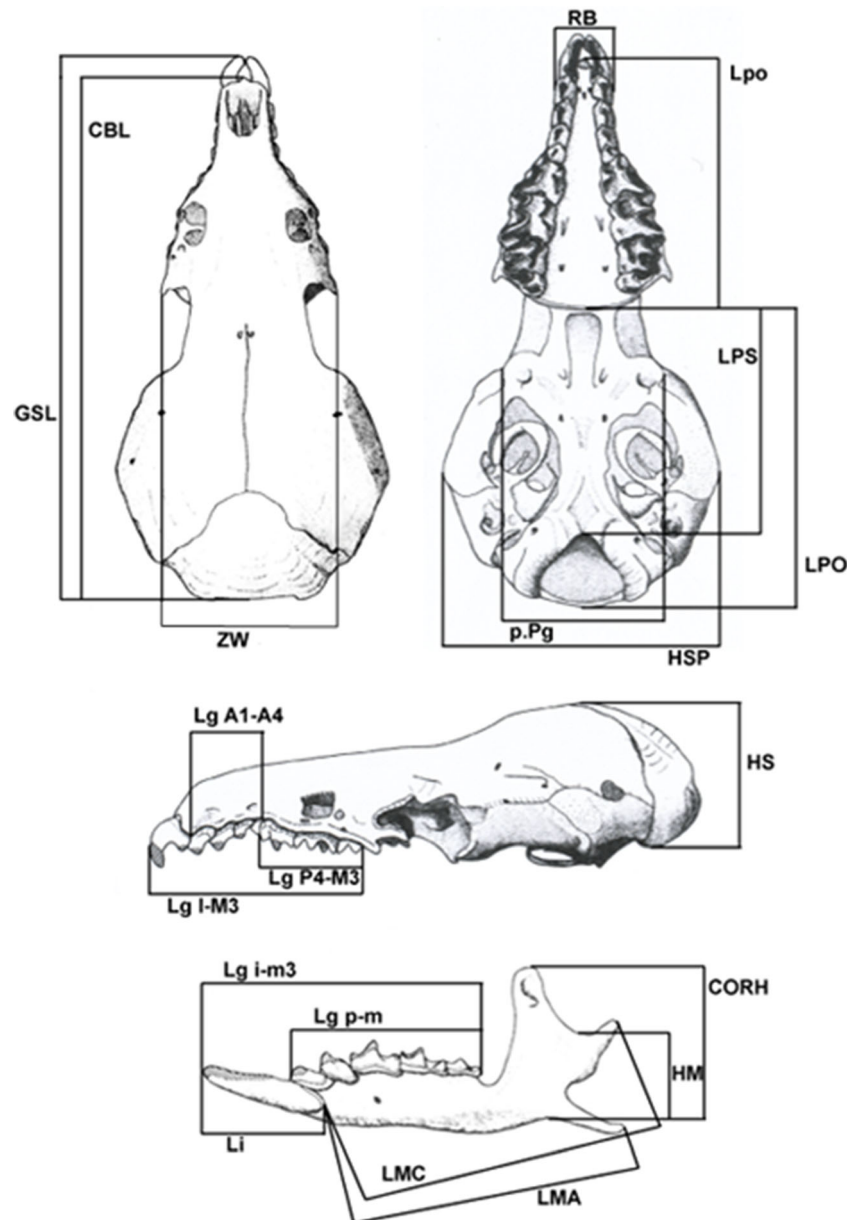
investigated due to their high information content, and foramina are usually the main non-metric characters. The appearance results primarily from genetic control (Berry [1975](#)) and is independent of growth and sex (Ansorge [2001](#)); wherefore, they are suitable for the assessment of the genetic variability and divergence among populations (Sjøvold [1977](#); Ansorge et al. [2012](#); Baker and Hoelzel [2013](#); Tibbetts [2013](#); Wiig and Bachmann [2014](#); Ranyuk and Ansorge [2015](#)).

For the epigenetic study, 89 specimens of *N. fodiens* were finally available based on their state of preservation (Table [3](#)). Due to the smaller sample size, only group MS, SOL, and OLHT were useful for the analysis. The selection of non-metric skull characters was based on earlier studies on *Sorex araneus* (Pankakoski and Hanski [1989](#); Wójcik et al. [2007](#)) and *Muscardinius avellanarius* (Ansorge et al. [2012](#)). Well-preserved skulls were scanned intensely for suitable foraminas. A total of 11 bilateral foramina as well as one unilateral were investigated

Table 1 Number of specimens of age class (AG) 1 and 2 only included finally in the analyses with the linear method (abbreviations of regional samples see Fig. [1](#))

N. fodiens	Number	N. anomalus	Number
BW	14	BW	7
MS	38	GP	9
HEP	13	HT	9
SOL	17	PSA	8
OLHT	15		

Table 2 Number of specimens of age class (AG) 1 and 2 only included finally in the analyses with the geometric method (abbreviations of regional samples see Fig. 1)

N. anomalus	Number	N. fodiens	Number
BW	Mandible 6	BW	Lateral 14 Ventral 15 Mandible 15
GP	Mandible 9	MS	Lateral 36 Ventral 35 Mandible 37
HT	Mandible 7	HEP	Lateral 9 Ventral 12 Mandible 12
PSA	Mandible 8	SOL	Lateral 13 Ventral 12 Mandible 18
		OLHT	Lateral 8 Ventral 8 Mandible 14

Table 3 Sample size for the epigenetic analysis of N.fodiens (abbreviations of regional samples see Fig. 1)

Regional group	Number
MS	49
OLHT	24
SOL	16

(Fig. 4). Bilateral traits were taken from both sides of the skull and registered separately.

A χ^2 test ($p = 0.05$) was used to furnish proof that there was homogeneity between the sex and the age structure and the frequencies of the character expressions. The initial analysis for homogeneity in age and sex of the non-metric characters revealed significant dependence. The χ^2 test exposed correlations between sex and the foramina B2 right ($\chi^2 = 5.306$, $p = 0.05$) and C1 left ($\chi^2 = 4.167$, p = 0.05) as well as correlations between age and foramen A2 right ($\chi^2 = 9.221$, $p = 0.05$). Therefore, these traits were excluded for further analysis.
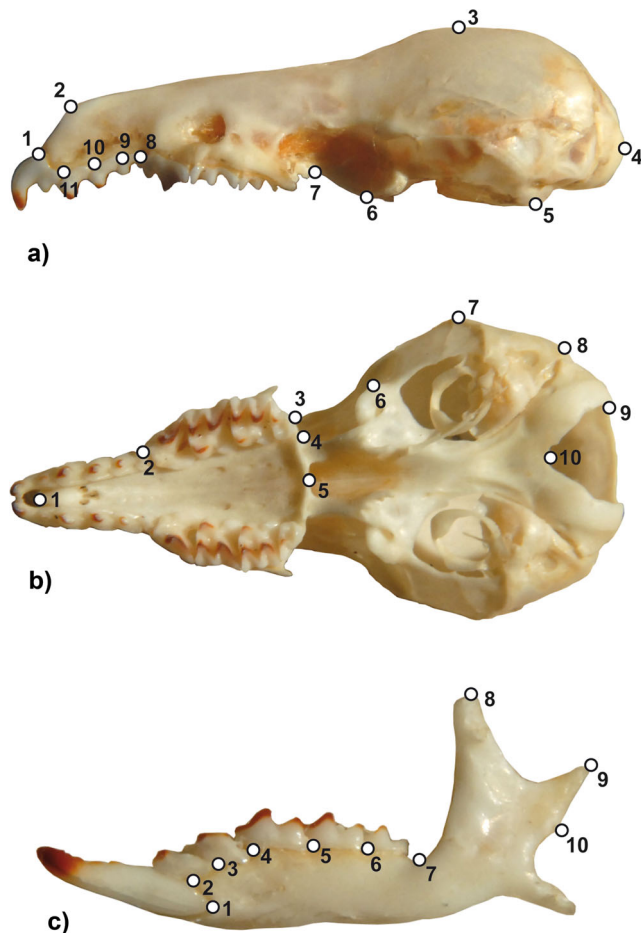
Fig. 3 Landmarks used to represent shape in **a** lateral view of skull, **b** ventral skull, and **c** in lateral view of mandible
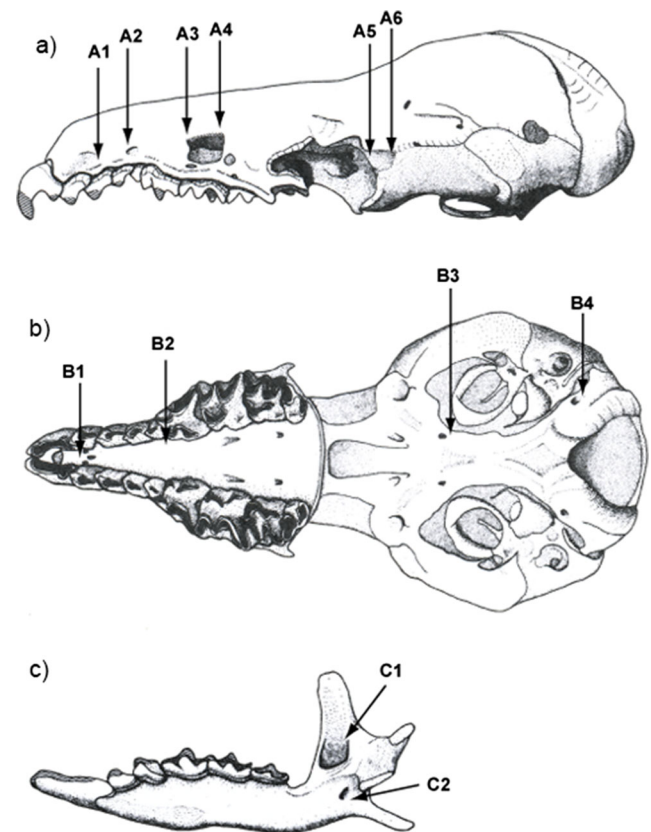
Fig. 4 Position of 12 non-metric characters in *Neomys fodiens* used for epigenetic study in lateral (**a**), ventral (**b**), side of skull and lingual side of mandible (**c**). (Drawings modified according to Spitzenberger (1990b)). *A1* anterior C^1 present; *A2* accessory foramen maxillare present; *A3* anterosuperior foramen infraorbitale present; *A4* posterosuperior foramen infraorbitale present; *A5* accessory foramen postorbitale I present; *A6* accessory foramen postorbitale II present; *B1* foramen incisivum anterior present; *B2* foramen palatine medial present; *B3* posterior foramen basisphenoid present; *B4* posterior foramen basioccipital present; *C1* foramen coronoidale present; *C2* accessory canalis radicis dentis present

The non-metric traits occured with different frequencies resulting in a single variability for every trait (Ansorge et al. 2012). The degree of the epigenetic variability I_{ev} for a population sample was calculated according to Smith (1981):

$$I_{ev} = 1 - \frac{\sum_{i=1}^{n} |50\% - F_i|}{n \cdot 50\%}$$

where n = number of characters and F_i = frequency of the ith character.

The degree of epigenetic distance was calculated by the widely applied and preferred "mean measure of divergence" (MMD) derived from the Mahalanobis distances (Sjøvold 1977):

$$\text{MMD} = \frac{1}{r} \sum_{i=1}^{r} \left[(\Theta_{1i} - \Theta_{2i})^2 \right] - v_i \quad S_{MMD} = \sqrt{\frac{2 \sum_{i=1}^{r} v_i^2}{r^2}}$$

where r = number of traits, n = sample size, p = frequency of traits, Θ = arcsin $(1-2p)$,

$$v_i = 1/n_1 + 1/n_2$$

The standard deviation (S_{MMD}) of the MMD indicated statistical significance at the level of $p = 0.05$ to be MMD > 2 S_{MMD}. All the groups were compared with each other by MMD calculations.

Futhermore, the fluctuating asymmetry (FA) was analyzed to measure the developmental stability (Ansorge 2001). A higher degree of asymmetry suggests lower stability and fitness. The degree of fluctuating asymmetry (FA) is defined as the relation of the number of asymmetric occurrences of a single character to the sample size. The unscaled mean of all characters results in the degree of asymmetry of the population (Palmer and Strobeck 1986).

Results

Linear morphometrics

The results of the Pearson correlation between *CBL*/*CORH* and altitude/latitude/longitude are given in Table 4 (for all results of linear measurements, see Online Resource 16–18). *CBL* was not statistically significant in both species with altitude, but CORH was negatively correlated in *N. fodiens* and positive in *N. anomalus*. Strong positive correlation of the *CBL* in *N. fodiens* and weaker negative correlation in *N. anomalus* with longitude were also found. In *N. fodiens*, *CORH* is not significant, but positively correlated in *N. anomalus*. No significant results between latitude and

Table 4 Pearson correlation coefficient of linear measurements *CBL* and *CORH* vs. altitude, longitude, and latitude for *N. fodiens* and *N. anomalus*

	Altitude	N	Longitude	N	Latitude	N
N. fodiens						
CBL	0. 076	80	0.323**	82	0.136	82
CORH	− 0.344**	92	0.081	95	0.264**	95
N. anomalus						
CBL	− 0.405	22	− 0.470*	22	− 0.153	22
CORH	0.365*	37	0.441**	37	0.648	37

N sample size

*The correlation is significant at a level of 0.05 (both sides); **the correlation is significant at a level of 0.01 (both sides)

CBL were found in both species and *CORH* was only positive correlated in *N. fodiens*.

The DA for *N. fodiens* from five regions yielded four functions and 40 of the 97 processed cases were valid. The eigenvalue = 36.186 of the first function was very high as well as the canonical correlation coefficient of 0.986. This suggests that the variance between the regional groups is clearly higher than within the groups. The criterion Wilk's lambda = 0.002 and the χ^2 test (χ^2 = 162.338, $p = 0.000$) confirmed a reliable separation of groups. The characters highly associated with function 1 are *Lpo* and *HM*. By means of group centroids, the mean values of the single functions can be compared (Fig. 5). The regional groups Baden-Württemberg (BW), Märkische Schweiz (MS), and Heppenheim (HEP) were obviously separated from the groups Southern Upper Lausitz (SOL) and Upper Lausitz Heath and Pond landscape (OLHT) by function 1 (mainly influenced by *Lpo* and *HM* indicating that the regional groups differ particularly between the length of the maxilla and the height of the mandible behind the coronoid) whereas function 2 (mainly influenced by *pPg*, *LgP4M3*, *LgIM3*, and *HSB*) separated the samples from Brandenburg (MS) and Southern Germany (BW, HEP).

In the DA for *Neomys anomalus*, 16 of the 33 processed cases were valid and yielded three functions and each with a high eigenvalue and canonical correlation coefficient (Fig. 6). This indicated a reliable explanatory model (Wilks lambda = 0.000, χ^2 = 60.988, $p = 0.006$). Correlation coefficients between the linear distances and the first two calculated functions showed that the major influence on the differentiation in regional groups seemed to be *LgA1A4*. The scatter plot illustrated a separation of three groups, in which the Saxony (PSA) appeared clearly separated from the Slowakian (HT) shrews by function 2 as well as from Southern Germany (BW, GP) by function 1.
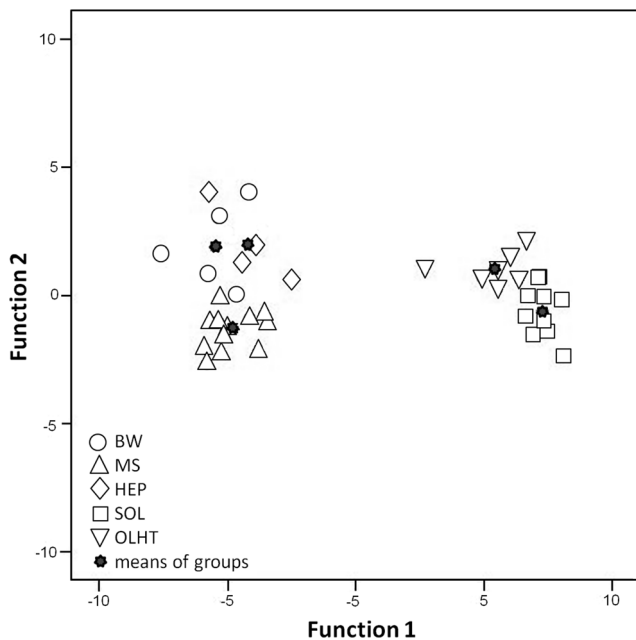
Fig. 5 Scatter plot of canonical discriminant analysis of linear measurements with 40 skulls from five regions of *N. fodiens* (others were automatically eliminated by SPSS because of lacking variables). The eigenvalue of Function 1 was 36.186 with a share of 90.1% of the total statistical variance. The canonical correlation coefficient accounted for 0.986 and Wilks' Lambda = 0.002; χ^2 = 162.338, p = 0.000. For Function 2, the eigenvalue amounted 2.013 with a share of 5%. The canonical correlation coefficient was 0.817 and Wilks' Lambda = 0.091; χ^2 = 64.708, p = 0.151). A total of 95.0% was correctly classified (abbreviations of regions see Fig. 1)
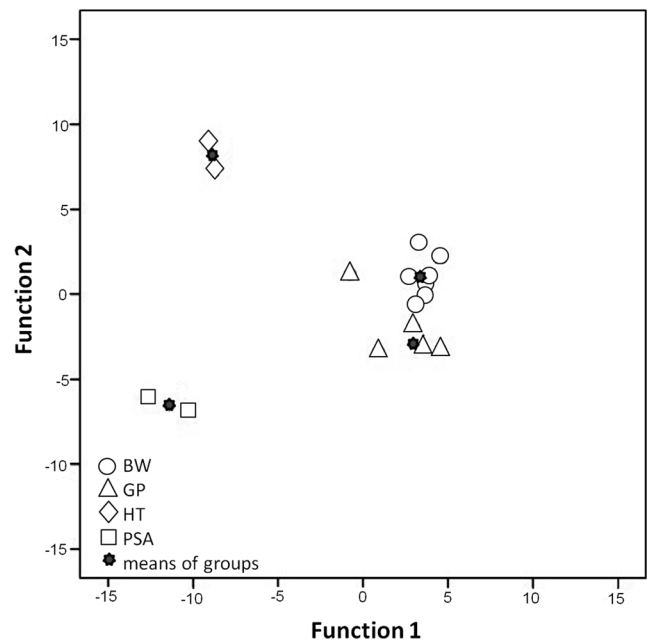

Fig. 6 Scatter plot of canonical discriminant analysis with 16 skulls from four regions of *N. anomalus* (others were automatically eliminated by SPSS because of lacking variables). For Function 1, the eigenvalue amounted 46.252 with a share of 63.7% of the total statistical variance. The canonical correlation coefficient was 0.989 and Wilks' Lambda = 0.000; χ^2 = 60.988, p = 0.006). The eigenvalue of Function 2 was 21.623 with a share of 23.8%. The canonical correlation coefficient accounted for 0.978 and Wilks' Lambda = 0.008; χ^2 = 34, p = 0.049. One hundred percent was correctly classified (abbreviations of regions see Fig. 1)

Geometric morphometrics

A principal component analysis was used to assess the shape variation. Along PC 1, a thin-plate spline illustrates the correlated shift in landmark points from one extreme to the other. Areas with bent grid demonstrate where the difference in skull morphology is the greatest. The thin-plate spline for *N. fodiens*, lateral view, described changes in the complete posterior skull area (Fig. 7a). The first PC accounted for 25.8% of the variance. The strongest shift exhibited the top of the os parietale in the anterior direction and a widening of the os temporale and the os occipitale. Also, the first PC of *N. fodiens*, ventral view, described shifts in the posterior area and accounted for 28.6% of the variance (Fig. 7b). The os temporale experienced a posteromedial extension and the os occipitale a ventral compression. Small deviations were present in the osseous parts of the rostrum. The first PC of the mandibular shape of *N. fodiens* (28.5% of the total variance) described a minimal magnification of the ramus mandibularis (Fig. 7c). The processus coronoideus inclined to an anteroinferior compression, whereas the processus condylaris described changes in inferior direction. Thus, processus condylaris and processus angularis approached each other. For *N. anomalus*, the PC 1 of the mandible shape (27.7% of the total variance) described similar strong variations for all

landmark configurations (Fig. 7d). The area around the incisivus characterized superior shifting and the molars changed in inferior direction. The variation of the ramus was less pronounced, but the processus coronoideus exhibited ventral modifications, and the processus condylaris and angularis experienced a small superior and dorsal shift.

To discover intraspecific differences between the regional groups by 2D landmarks, a discriminant analysis was used, too. For *N. fodiens*, lateral view, the analysis resulted in four functions that together accounted for 100% of the variance, of which 65.92 (eigenvalue = 2.99) can be attributed to function 1. Procrustes and Mahalanobis distance were calculated for each regional group. Major distances arose for the group pair HEP/OLHT, and so the variability between those two was the highest, but the group means were not statistically significant (T^2 = 357.33, p = 0.5602). There was also a high Procrustes distance and a middle-high Mahalanobis distance between group HEP and MS (T^2 = 249.11, p < 0.0001). Between them, the corresponding landmark positions and group centroids were pronounced similarly. With decreasing distances follow the group pairs: HEP/SOL (T^2 = 438.46, p = 0.1564), BW/HEP (T^2 = 399.99, p = 0.0858), and BW/SOL (T^2 = 150.36, p = 0.0792). In contrast, small distances were observed between the groups BW/OLHT (T^2 = 101.55, p = 0.6554), MS/SOL (T^2 = 160.06, p < 0.0001), BW/MS (T^2 = 117.60,

Fig. 7 Variation along first
principal components of shape of
Neomys fodiens (**a**–**c**) and
Neomys anomalus (**d**). **a** Lateral
skull landmarks. **b** Ventral skull
landmarks. **c**, **d** Lateral mandible
landmarks
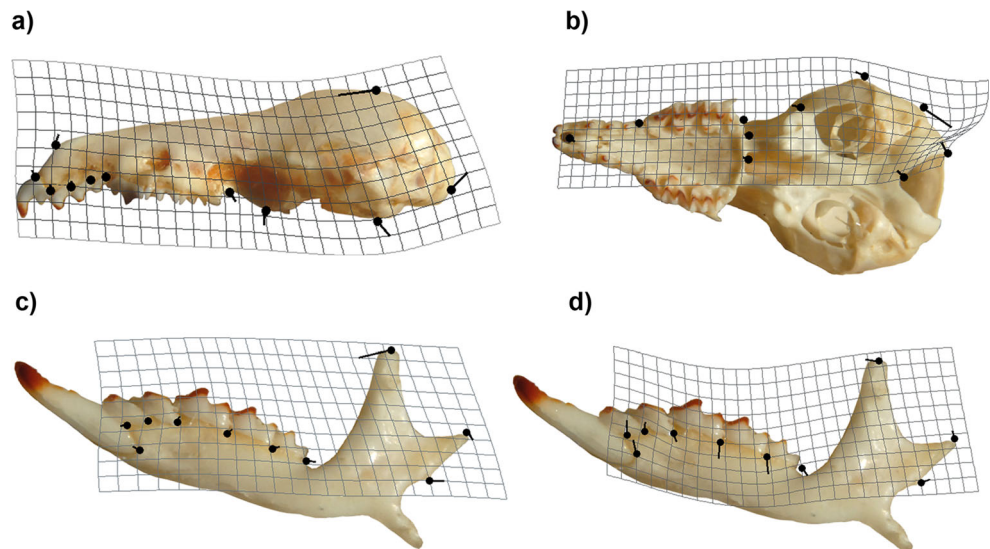
p = 0.0002), and MS/OLHT (T² = 59.50, *p* = 0.0585), respectively. The scatter plot revealed the scores of the five water shrew groups for the first and second functions (see Fig. 8). No clearly separated groups could be recognized, just tendencies.

The discriminant analysis for the ventral view of the skull of *N. fodiens* yielded four functions; function 1 accounted for 77.10% of the total variance (eigenvalue = 2.85). The highest Procrustes distance was due to group BW/MS, and the Mahalanobis distance was also high (T² = 257.90,

p < 0.0001). The groups MS/SOL (T² = 89.33, *p* = 0.0009) and MS/OLHT (T² = 41.20, *p* = 0.1292) showed high Procrustes distances with small Mahalanobis distances. In contrast, the regional group pairs HEP/OLHT (T² = 221.81, *p* = 0.2676) and BW/OLHT (T² = 182.52, *p* = 0.0758) had high Mahalanobis distances with medium Procrustes distances. Inconspicuous values were calculated for the remaining groups. Thus, the shape differences were smaller between geographically closer regions. Again, there was no clear separation in the cloud diagram for the water shrew groups
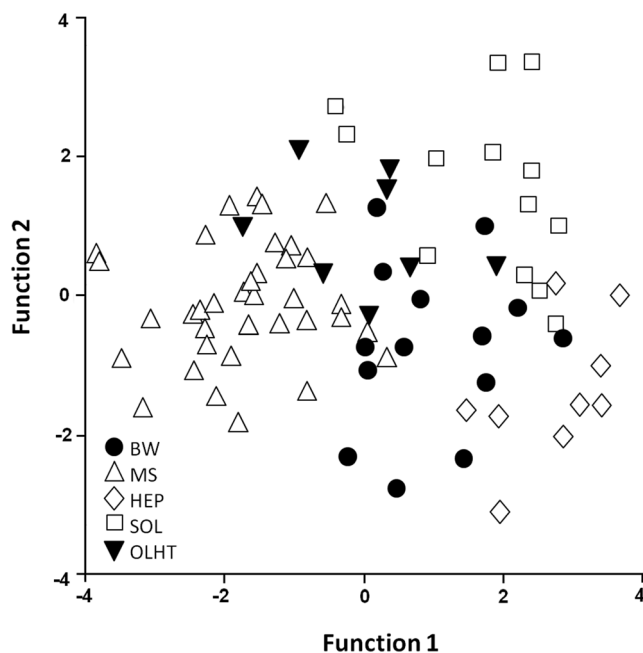
Fig. 8 Scatter plot of discriminant analysis from 5 regions of *N. fodiens*—lateral view with 80 skulls. The eigenvalue of Function 1 was 2994 with a share of 65.92% of the total statistical variance. For Function 2, the eigenvalue amounted 0.857 with a share of 18.9% (abbreviations of regions see Fig. 1)
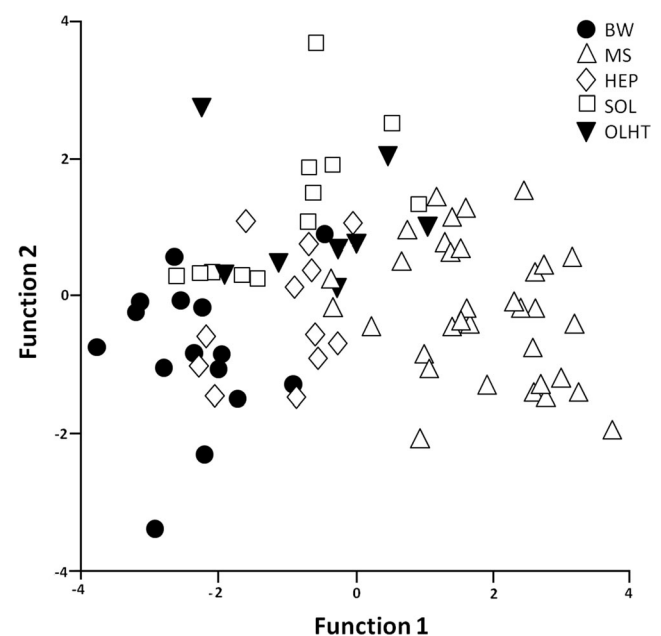
Fig. 9 Scatter plot of discriminant analysis of linear measurements from five regions of *N. fodiens*—ventral view with 82 skulls. The eigenvalue of Function 1 was 2.851 with a share of 77.1% of the total statistical variance. For Function 2, the eigenvalue amounted 0.529 with a share of 14.3% (abbreviations of regions see Fig. 1)

(Fig. 9). But the Baden-Württemberg samples appeared separated from the Brandenburg samples. Especially, the last one had relatively fewer overlaps with other groups.

For the lateral view of the mandible of *N. fodiens*, four discriminant functions were calculated whereby the first one accounted for 64.49% of the total variance (eigenvalue = 1.55). High values of Procrustes and Mahalanobis distances were observed for the farther away regional group pair MS/HEP (T^2 test = 98.36, $p < 0.0001$) and BW/MS (T^2 = 124.60, p < 0.0001), whereas the values were smaller between group pairs like BW/SOL (T^2 test = 34.55, $p = 0.4154$) and SOL/OLHT (T^2 = 32.44, $p = 0.4917$) with growing geographical distances. The scatter plot provided a diffuse distribution of the scores with many overlaps between the regional groups (Fig. 10). Mainly the water shrews from Brandenburg appeared separated.

For *N. anomalus*, the discriminant analysis calculated three functions. The first one accounted for 71.56% of the total variance (eigenvalue = 12.87). The Mahalanobis distances were extremely high, especially between GP/PSA (T^2 test = 5905.75, $p = 0.1521$) and GP/HT (T^2 test = 1042.01, $p = 0.3289$), whereby Procrustes distances were medium-size or rather low. The highest Procrustes distance value achieved group BW/HT (T^2 test = 144.25, $p = 0.6206$). Small Procrustes and Mahalanobis distances were present between the group pair BW/GP (T^2 test = 92.94, $p = 0.7995$), thus between the regions with the least regional distance. The T^2 test showed no significances of the difference between group means. In the scatter plot, there were three clearly well-separated groups (Fig. 11). Especially the Slovakian and the Saxon, shrews could be recognized as discrete groups. The regionally close samples from Southern Germany showed many overlaps.

Comparison of the results of the craniometric methods

For *N. fodiens*, the results of both methods differed partially. The linear method provided a more effective separation of the regional groups and thus a better model validity. Nevertheless, both methods identified morphological differences which increased mostly with growing geographical distance. The methods, however, indicated different characters, in which the groups were distinct: the ventral skull area and the height of the mandible in linear measurements compared to the posterior skull area and the ramus mandibulae in GMM.

For *N. anomalus*, the model validities in both methods were relatively good, and the scatter plots illustrated clearly separated regional groups, respectively. Thus, both methods verified the geographical differences. But because only the mandible shapes were useful for examination in the geometric method, the main separating traits can only be compared for that bone structure. The GMM showed variations for all landmarks with a clear shift between the premolar and molars, whereas in the linear analysis, the strongest variations in the mandibular seemed to be related to the ramus mandibulae (but the factor loadings are quite smaller compared to the other linear measurements like LgA1A4).

We tested also for "isolation by distance" using a linear regression. All calculations showed a positive relation between morphometric and geographic distance, but R^2 was always very low or rather there is only a small linear relation.

Epigenetic study

The average epigenetic variability of the examined samples of all the *Neomys fodiens* groups was $I_{ev} = 0.42$ (Table 5). The groups MS and SOL showed a similar variability, and group OLHT clearly showed less.

The epigenetic distances were quite small, and none of the pair-wise comparisons revealed any significance (Table 6). Even a comparison between the entire Upper Lausitz (OLHT + SOL) and MS provided no significant result.

In the eight traits investigated, asymmetries occurred for the regional groups, except for trait "A6," which always occurred symmetrical for the water shrew-group OLHT. Obviously, the degree of fluctuating asymmetry increased in northward direction. It was 15.3% in the southern Upper Lausitz, but in Brandenburg, it was clearly higher with 21.3% (Table 7).
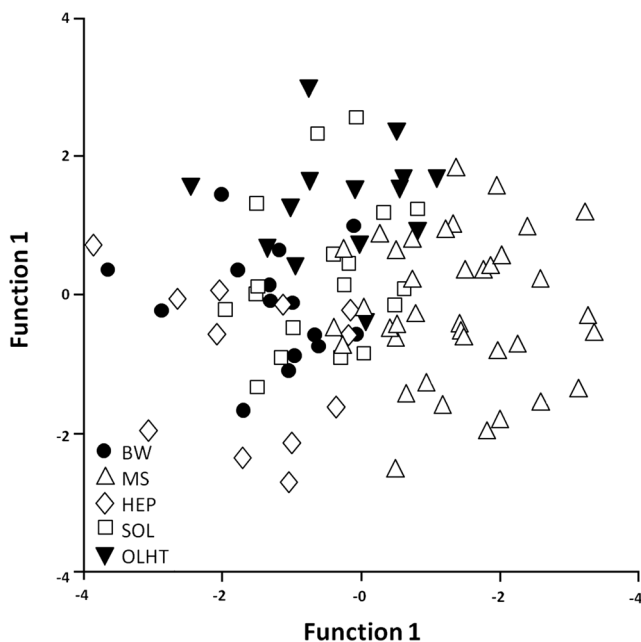

Fig. 10 Scatter plot of discriminant analysis from five regions of *N. fodiens*—lateral mandible view with 96 skulls. The eigenvalue of Function 1 was 2548 with a share of 64.5% of the total statistical variance. For Function 2, the eigenvalue amounted 0.451 with a share of 18.8% (abbreviations of regions see Fig. 1)

Fig. 11 Scatter plot of discriminant analysis from 4 regions of *N. anomalus*—lateral mandible view with 30 skulls. The eigenvalue of Function 1 was 12.865 with a share of 71.6% of the total statistical variance. For Function 2, the eigenvalue amounted 2.625 with a share of 14.6% (abbreviations of regions see Fig. 1)
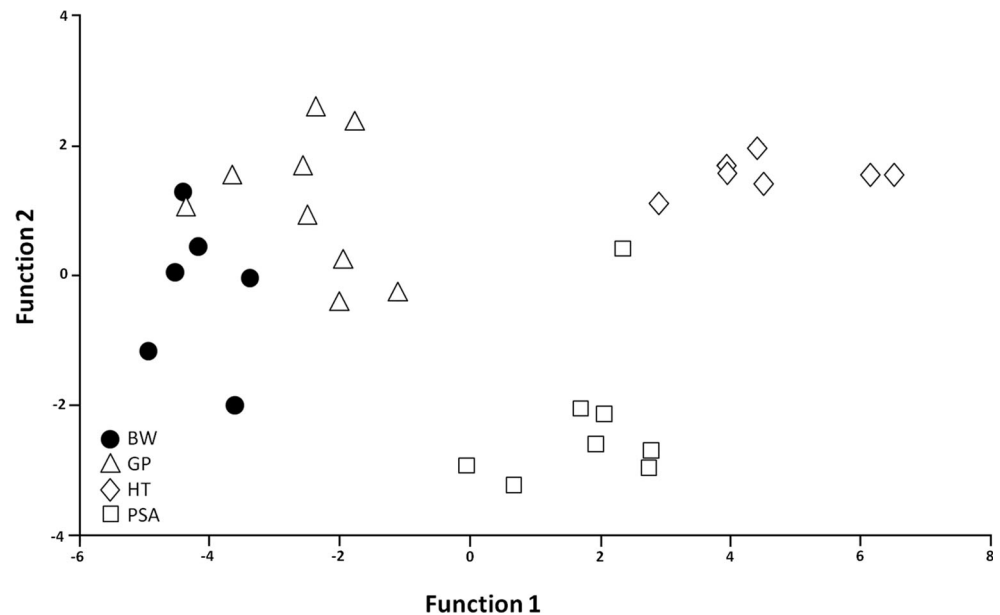
Discussion

Craniometric morphometry

The craniometrical study revealed intraspecific geographical differences in the skull of *Neomys fodiens* and *N. anomalus* differently pronounced in both methods.

For the *N. fodiens*, the linear method revealed that especially the groups BW, HES, and MS were separated from the eastern German groups SOL and OLHT. Thus, the Saxon skulls showed distinct morphological differences to those from Brandenburg and Southern Germany. The shrews from Brandenburg were less separated from the farther away samples from Southern Germany than from the (geographically) closer Saxonian samples. So, the variations cannot only be attributed to geographical distances. Likewise, other authors were unable to find a geographical trend (Niethammer 1960; López-Fuster et al. 1990; Kryštufek and Quadracci 2008). Spitzenberger (1980) investigated *Neomys*-species in Austria and assumed the variations in size to be the result of ecological factors such as climatic conditions and the accompanying changes in seasonal food supply as there were clear altitudinal differences. The studied material here originate only from

lower altitudes (up to about 700 m only), and there was no correlation between *CBL* such as an indicator of overall body size (Ochocińska and Taylor 2003), and altitude. Price (1953) noted that water shrews from moist biotopes weighed more than those from arid biotopes; unfortunately, local conditions could no longer be assessed for the collection material used in this study. Spitzenberger (1990b) describes that the skull measurements *CBL* and *CORH* are smaller from North Germany till Scandinavia as in southern regions. In our study, the mean values of the measurements *CBL* and *CORH* increased from south to north and thus followed Bergmann's rule. The positive correlation between *CORH* and latitude verified this.

Rychlik et al. (2006) noted that there is a correlation between the geographical variation in size and presence/absence of the twin species *Neomys anomalus* in Poland, which potentially arises from a competition between the species. When living in allopatry, there was also an accordance with Bergmann's rule, which might apply here as well.

Also, the geometric discriminant analysis for *N. fodiens* definitely showed geographical differences, but not as clearly, and the model validity with lower eigenvalues was moderate. The Procrustes distances and the Mahalanobis distances tended to be higher between groups with greater geographical

Table 5 Epigenetic variability I_{ev} of the *Neomys fodiens* samples (abbreviations of regional samples see Fig. 1)

Regional group	Epigenetic variability I_{ev}
MS	0.43
OLHT	0.34
SOL	0.48
∅	0.42

Table 6 Mean measure of divergence (MMD) between the *Neomys fodiens* groups and their standard deviations (SD) (abbreviations of regional samples see Fig. 1)

Regional group pair	MMD	SD
MS-OLHT	0.02374030	0.0457252
MS-SOL	0.01119500	0.0291578
OLHT-SOL	0.06122970	0.0532888
MS-total Upper Lausitz	0.00657907	0.0227671

Table 7 Fluctuating asymmetry FA (%) in the *Neomys fodiens* samples (abbreviations of regional samples see Fig. 1)

Regional group	FA
MS	0.213
OLHT	0.188
SOL	0,153

distances, but this is not the rule, and there is also no clear correlation between variance of shape and regional distance. The biplots reflect this with no clear separation of groups and only tendencies toward grouping, with the Brandenburg sample showing the least overlaps. This might be explained by an actual, distinctive difference to the other groups, supported by high Procrustes and Mahalanobis distances. The skulls were in a very good state, which simplified the positioning of the landmarks and led to exact measuring points, benefitting the analysis (Zelditch et al. 2012). Another reason might have been the relatively large sample size of this group ($N = 45$ per configuration, other groups had mostly fewer than 20).

The linear method indicated the ventral skull area (*Lpo*) and the height of the mandible (*HM*) as the mean separating traits for distinguishing the water shrew groups. Also, the thin-plane spline illustrates variations between the shapes in the geometric analysis, but partly contrary to the linear method. The water shrews differed here preponderantly by means of the posterior skull area, particularly in the length of the os parietale and the os frontale and in the extension os temporale and os occipitale. The mandible also showed variations, but particularly for the ramus mandibulae.

Using linear measurements, three regional groups can be clearly separated for the *Neomys anomalus* samples (BW/GP, HT, and PSA). Hence, there might be a correlation between morphology and geographical distance as well. Spitzenberger (1990a) used *CBl-* and *CORH*-data from different studies to describe geographic variations of skull measurements in *N. anomalus*. As a result, both measurements decreased from south to north and from east to west. Furthermore, skulls are larger in the East European lowland than in the Alps and upstream low mountain range. Though, in our study, the Pearson correlation for *CORH* was positively correlated with altitude and there were no significant results between *CBL* or *CORH* and latitude, but therefore with longitude. *CBL* was negatively correlated and reflects Spitzenberger (1990a) observations, but the positive correlation with *CORH* gives a heterogeneous result. Kryštufek and Quadracci (2008) found also an increase in body size that contradicts Bergmann's rule; allopatric samples were larger than sympatric populations, which is consistent with the hypothesis of character displacement. In Poland, *N. anomalus* followed Bergmann's rule when they occurred in allopatry (Rychlik et al. 2006). In sympatry, they tended to have greater variations than *N. fodiens*. The possible reason for the greater variation might have been stress due to intraspecific competition with the dominant

water shrews. Highly aggressive potential of *N. fodiens* was confirmed by other studies (Krushinska and Rychlik 1993; Krushinska et al. 1992, Rychlik and Zwolak 2005). Research revealed that stress reduces developmental stability and promotes environmentally phenotypic variations (Zakharov et al. 1991; Ansorge 2001). Therefore, the inconsistent results may ensure from the sympatry with *N. fodiens*. Another aspect to consider by discussing the reasons of the greater morphological variation of *N. anomalus* is the fact that the compared regions of *N. anomalus* were father apart (especially group HT from Slovakia) than in *N. fodiens*. Furthermore, the distribution range of both species is generally different. Whereas *N. anomalus* distribution is more scattered, the populations of *N. fodiens* are more continuous and numerous (Kraft 2008). This could lead to a better exchange between individuals of *N. fodiens* and thus to less differences between regional groups.

Likewise, the landmark-based method yielded a good separation of three regional *N. anomalus* groups and showed a correlation between shape and location. However, the sample size was quite small, and only the mandible shapes could be analyzed. Thus, conclusions for the whole skull must remain sheer speculation; nevertheless, there is a connection between dentition, body, and prey size for shrews (Popov and Zidarova 2008). Due to the very distinct separation of far distant groups and overlapping of near distant groups, respectively, a geographical variance of *N. anomalus* seems very probable.

The linear method indicated *LgA1A4* as the mean separating trait. Thus, *N. anomalus* are distinguished mostly by the length of the unicuspid teeth of the maxilla, whereby the values of the Slovakian group are remarkably small. Rychlik et al. (2006) reported similar observations in Poland and traced this to dietary options. Different authors described correlations between body and prey size (Churchfield and Sheftel 1994; Dickmann 1988), bite force and skull length (Carraway and Verts 1994), and different lengths of incisives between the *Neomys* species (Popov and Zidarova 2008). So perhaps, the Slovakian shrews preferred smaller prey. Thus, the morphological differences may not only be caused by physical, but also by biotic factors.

Epigenetic investigation

The analysis of non-metric traits mainly serves the investigation of the isolation problems of endangered species and the assumed accompanying impoverished gene flow (Pertoldi et al. 2000; Ansorge et al. 2012; Ranyuk and Monakhov 2011; Ranyuk and Ansorge 2015). Indeed, water shrews have a wide distribution range in Germany, but they are under special protection and included on the early warning list of the German Red List. To see if there is a potential genetic depression, the degree of epigenetic variability I_{ev} gives implications for conservation measures.

For all the *Neomys fodiens* together, the epigenetic variability of $I_{ev} = 0.42$ was quite high. So, there is no indication of a possible reproductive isolation. All three regions exhibit high values, whereby the samples of group OLHT showed the smallest value. Maybe this is a reflection on the reclamation of the pond landscape and the corresponding human impact, which started already in the thirteenth century (Bastian et al. 2005). Likewise, for another eulipotyphla, the *Talpa europaea*, Ansorge (1994) obtained a very high epigenetic variability of $I_{ev} = 0.34$ whereas other studies of small mammal species mostly rendered smaller variabilities (Markov 2003; Uhlikova 2004).

The degrees of the epigenetic distances between the water shrew groups were very small and revealed no significances, which indicates a minor separation. Small distances were also found for *Sorex araneus* (MMD = 0.0135–0.0426) (Wójcik et al. 2007) and for *Talpa europaea* (MMD = 0.05–0.13) (Ansorge 1994). In general, other small mammals (e.g., rodentia) exhibited major distances between their populations (Ansorge et al. 2012; Uhlikova 2004; Markov 2003).

In addition, the non-metric traits were examined in reference to the deviations from bilateral symmetry. The resulting fluctuating asymmetry (FA) measures the developmental stability (Badyaev et al. 2000; Zakharov et al. 1991; Wójcik et al. 2007; Ansorge 2001) and reflects the degree of environmental or genetic stress (e.g., mutation, inbreeding, hybridization) an individual is exposed to during ontogenesis (Tomkins and Kotiaho 2001). According to Lazarová (1999), FA varies in different small mammal species from FA = 0.14 to FA = 0.44. In the water shrew groups, the degree of asymmetry was within this range, but, compared with other mammals, in the lower array (Fa = 0.15–0.21). Thus, there is only a small indication of reduced developmental stability in all three regional groups, and a geographical trend is visible as well as the FA increased from south to north. The asymmetry is distinctly more scarce in the southern Upper Lausitz than in Brandenburg. Some of the possible causes may have been different ecological conditions or disturbances which led to stress. In Poland, Wójcik et al. (2007) found correlations between asymmetry and habitat quality for three populations of *Sorex araneus*. For the water shrew collection material, the precise habitats and likewise the ecological conditions are not comprehensible. In fact, the groups originated from the same region, but their exact collection sites were scattered over a larger radius. So, the habitats may have varied already within the sample itself. Stress can also be caused by intra- or interspecific competition and have a destabilizing effect (Wójcik et al. 2007; Zakharov et al. 1991). Thus, possibly the higher FA of the Brandenburg group resulted from stronger interspecific competition with *Neomys anomalus* or other Soricidae. Tomkins and Kotiaho (2001) mentioned inbreeding and go-along reduced genetic variance and fitness as another cause for asymmetry expressions. The possibility of an

isolated position of the Brandenburg group has already been mentioned. Therefore, various reasons may have contributed to asymmetries. However, in itself fluctuating asymmetry must be considered critically. A number of studies found no connection between the degree of fluctuating asymmetry and environmental conditions (Tomkins and Kotiaho 2001; Gilligan et al. 2000; Ansorge et al. 2012) nor to any analyses of genetic diversity (White and Searle 2008).

Conclusion

The present study confirms regional intraspecific variances for *Neomys fodiens* and *N. anomalus*. Both, the linear and the geometric morphometric method showed morphological skull differences, which usually increase with geographical distance. Especially, the results for *N. anomalus* yielded clearly separated regional groups. For *N. fodiens*, only the linear method revealed clear group separations, leading to the assumption that they differ more strongly in size than in shape. The attributed phenotypic variance caused by altitudinal conditions cannot be confirmed as several biotic factors seem to be of influence here.

There is no evidence of genetic depression and the degree of the epigenetic distances is small like in other eulipotyphla. We found a small indication of reduced development stability in all regional groups, which increased from south to north.

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