

Does the geometric and linear morphometry of the brain reflect the divergence in the “guentheri” group (Arvicolinae, *Sumeriomys*)?

Tanya Zorenko*, Ugis Kagainis & Lyubova Barashkova

ABSTRACT. The purpose of this work is to investigate the divergence of the brain's size and shape of three vole's taxa of “guentheri” group using geometric and linear morphometric analysis. The obtained data show that the sex factor does not affect the brain's linear parameters and shape of the studied taxa that indicates a weak effect of the sexual selection on morphological adaptation and variability of the brain structure. The linear measurements of the investigated taxa differ significantly by all signs (24 parameters). The obtained data indicate that the divergence of *Microtus guentheri* and *M. hartingi* is the most significant (92%) that obviously is associated with ancient isolation of this species by Anatolian Diagonal. The differences between the two subspecies (*M. hartingi hartingi* and *M. h. lydius*) are much smaller — 63%. It should be noted that the vole of the Strandzha Mountain — *M. h. strandzensis* differs from the *M. h. hartingi* and *M. h. lydius* significantly more — 82 and 75%, respectively. These differences may reflect the history of the investigation of voles from Anatolia to Europe. Geometric deformities of the brain of all taxa are found. Analysis of canonical variations showed that all three taxa differ significantly, both laterally and dorsally. Comparison of taxa lateral views showed that *M. h. lydius* brain is more flatten than *M. h. hartingi*, but *M. guentheri* has a broader brain and a smoother outer olfactory tract as well as smaller olfactory bulbs. By dorsal view, deformation of *M. guentheri* brain at the attachment sites of the cerebellum hemisphere to the cerebral hemisphere is observed, while the cerebellum worm of *M. h. lydius* is more flattened. The factors affecting the diversity of brain complexity could include allometry, phylogeny and natural selection. The accelerated morphological evolution of the “guentheri” group can be explained by the fragmentation of the range and habitats.

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Отражает ли геометрическая и линейная морфометрия мозга дивергенцию в группе “guentheri” (Arvicolinae, *Sumeriomys*)?

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РЕЗЮМЕ. Целью данной работы является исследование дивергенции размера и формы мозга трех таксонов полевок группы “guentheri” с использованием геометрического и линейного морфометрического анализа. Полученные данные показывают, что фактор пола не влияет на линейные параметры и форму мозга изучаемых таксонов, что свидетельствует о слабом влиянии полового отбора на морфологическую адаптацию и изменчивость структуры мозга. Линейные измерения исследуемых таксонов существенно различаются по всем признакам (24 параметра). Полученные данные свидетельствуют о глубоком расхождении *Microtus guentheri* от *M. h. lydius* и *M. h. hartingi* (92%), что, очевидно, связано с более древней изоляцией этого вида в результате образования горного хребта Анатолийской диагонали. Различия между двумя подвидами (*M. hartingi hartingi* и *M. h. lydius*) значительно меньше — 63%. Следует отметить, что полевка, обитающая в горах Странджа — *M. h. strandzensis* отличается от *M. h. hartingi* и *M. h. lydius* значительно больше — 82 и 75% соответственно.

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Эти различия могут отражать историю расселения полевков из Анатолии в Европу. Обнаружены также геометрические изменения мозга у всех таксонов. Анализ канонических вариаций показал, что все три таксона значительно различаются как в латеральном, так и в дорзальном направлении. Сравнение мозга сбоку у трех таксонов показало, что мозг *M. h. lydius* более плоский, чем *M. h. hartingi*, тогда как *M. guentheri* имеет более широкий мозг и более гладкий внешний обонятельный тракт, а также более мелкие обонятельные луковицы. С дорсальной стороны наблюдается деформация мозга *M. guentheri* в местах прикрепления полушария мозжечка к полушарию головного мозга, в то время как червь мозжечка у *M. h. lydius* более уплощен. Факторы, влияющие на разнообразие строения мозга, могут включать аллометрию, филогению и естественный отбор. Ускоренная морфологическая эволюция полевков группы “*guentheri*” может быть объяснена фрагментацией ареала и биотопов.

КЛЮЧЕВЫЕ СЛОВА: геометрическая морфометрия, структуры головного мозга, величина и форма мозга, морфологическая дивергенция, географическая изоляция, *Microtus guentheri*, *Microtus hartingi*.

Introduction

The “*guentheri*” group previously included one polytypic species — the Gunter’s vole *Microtus guentheri* Danford et Alston, 1880 with a karyotype of $2n = 54$ (Golenishchev *et al.*, 2002), which occupied a wide range from Israel, Lebanon, Syria to the Balkans and Africa (Niethammer & Krapp, 1982; Musser & Carleton, 2005; Gromov & Erbaeva, 1995; Kryštufek & Vohralik, 2005). The composition of the subspecies of the Gunter’s vole is not precisely defined. Six-seven subspecies were noted, of which three in Europe (Gromov & Polyakov, 1977). However, genetic molecular studies with the use of mt-cyt *b* marker showed that *M. guentheri* is present in southeastern Anatolia, but in western Anatolia and Europe (Thrace) is *Microtus hartingi* Barret-Hamilton, 1903 (Kryštufek *et al.*, 2009; Thanou *et al.*, 2012). Both species are isolated from each other by a mountain range (Anatolian diagonal) (Yiğit & Çolak, 2002).

M. hartingi lives in central and northwestern Anatolia and the Balkans. However, populations from Thrace and western Anatolia have long been isolated. The Straits of Dardanelle and the Bosphorus were formed in the late Pliocene about two million years ago (Çağatay *et al.*, 2000; Yaltırak *et al.*, 2000), and some authors believe that the strains closed the gene flow between Asia and Europe, and the species formation has intensified (Yiğit & Çolak, 2002; Yiğit *et al.*, 2012).

The status of voles from Europe is still not clearly defined. According to molecular data, there is no reason for the European populations of *M. hartingi* to consider as an independent species (Yiğit *et al.*, 2017). However, the history of settlement and the formation of the *M. hartingi* range in Europe indicates the specificity of the existence of populations under conditions of fragmented habitats (Kryštufek *et al.*, 2018), which could affect both the morphological and behavioural characteristics of European populations.

Significant differences in the morphology of the voles from western Anatolia and Thrace were found. Based on differences in back colouration, the shape of the baculum, and some exterior features, a number of authors suggested that southeastern populations could be considered as an endemic species of western Anatolia. *M. lydius* Blackler, 1916 differs from *M. hartingi* from

southeastern Thrace (Bulgaria and European Turkey) (Yiğit & Çolak, 2002; Yiğit *et al.*, 2012; Markov *et al.*, 2014). Differences in the linear parameters of sperm (Zorenko & Golenishchev, 2015) and cytogenetic differences between these populations also were found (Zima *et al.*, 2013). At the molecular level, differences between voles from Anatolia and Thrace were also identified: the index of nucleotide diversity and number of mutations in the three genes (mt-co1, mt-cytb, 12S rRNA) of *M. hartingi* from Thrace are very low in comparison to a higher values of these indicators in the voles from western Anatolia (Cetintürk, 2018).

Hybridization of voles from Anatolia and Thrace revealed a high level of male sterility. Although F1 hybrids are quite viable and can breed, their descendants (F2 and backcross) are often unviable, subvital, semi-sterile or sterile, which is the result of a “hybrid breakdown” (Zorenko *et al.*, 2016).

The brain is the important structure of animals. The brain has been studied in many mammalian species (Belenkov & Goreva, 1969; Khrustaleva *et al.*, 1994; Voino, 1994; Zorenko, 2013), but relatively few studies have been devoted to a group of closely related species and genera (Kovalenko, 1982). The results of investigations of the external structure of the brain were used in functional morphology (Andreeva & Obukhov, 1999; Bruner *et al.*, 2012, 2014), as well as in systematic (Zorenko, 2013) and evolutionary studies (Bruner, 2004). In this regard, not only linear measurements but also geometric morphometrics to describe morphological variation of various indices can be used (Pavlinov & Mikeskina, 2002; Barčiová, 2009; Voyta *et al.*, 2013; Kamilari *et al.*, 2013; Gorodilova & Vasilyeva, 2014). Physicians (Boyagina *et al.*, 2016) and anthropologists (Bruner, 2004; Bruner *et al.*, 2012, 2014) show the considerable attention to the study of geometric morphometric of the brain. The brain size and shape may have played a relevant role in human brain evolution (Bruner *et al.*, 2010). Therefore, using of the brain in study of closely related species of rodents can open up new perspectives in understanding of their evolution.

The purpose of this work is to investigate the divergence of the brain’s size and shape of three taxa of “*guentheri*” voles group using linear and geometric morphometric analysis.

Material and methods

This work was carried out using the collection of animals from the Laboratory of Ethology (University of Latvia). The voles, in total 60 (F_1 – F_7 generations, aged 6–7 months) were collected from three different locations (Fig. 1). Each location represented one taxon of the “guentheri” group: *M. guentheri* (4 ♀ and 8 ♂ from Türkoğlu/Kahramanmaraş, Turkey), *M. h. hartingi* (12 ♀ and 12 ♂ from Rhodope Mountains, Bulgaria), and *M. h. lydius* (12 ♀ and 12 ♂ from the vicinity of Kırşehir, Turkey). We accept that the voles from the Rhodope belong to the *M. h. hartingi* subspecies, since they border on the Greek populations. Earlier we

performed linear morphometry of *M. h. strandzensis* (Zorenko, 2013); however, a comparison was made with taxa of the “socialis” group. Therefore, in this study we used data on this subspecies for comparison with taxa of the “guentheri” group.

The conditions of keeping and working with animals were compiled in accordance with standards of Council of the European Communities 86/609/EES. Animals were sacrificed by cervical dislocation, which is regarded as a humane method for small rodents (FELASA, certificate C-category). The skull with the brain was placed in neutral 10% formalin (for 10 days). Then the brain was removed from the skull and fixed in formalin.

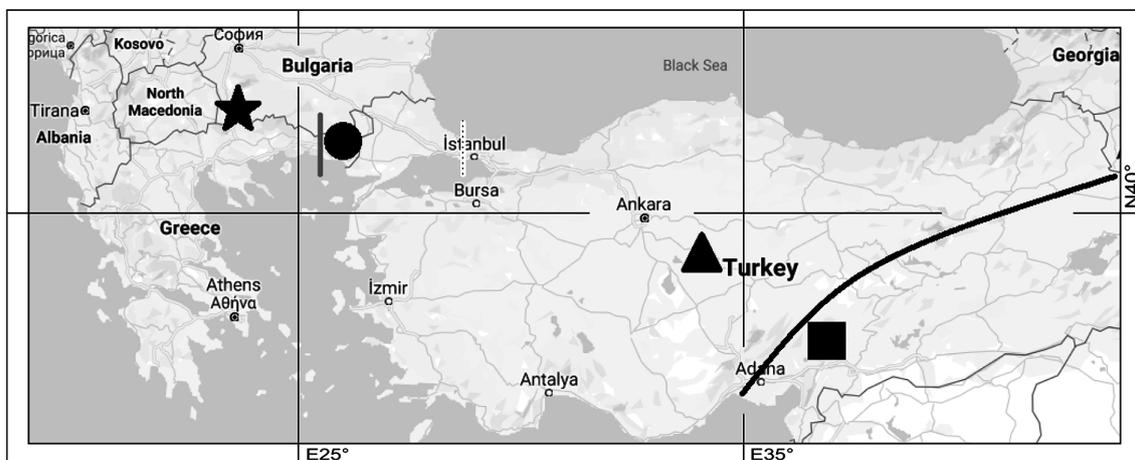
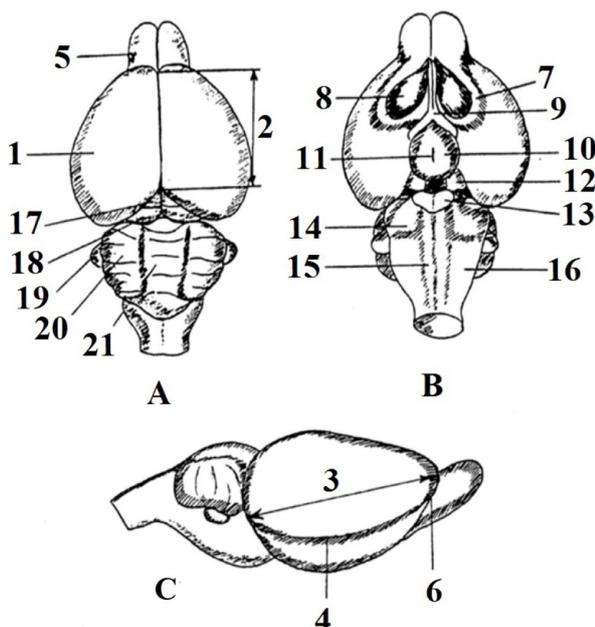


Fig. 1. “Guentheri” group taxa studied locations: star — Rhodope (*M. h. hartingi*), circle — (*M. h. strandzensis*) (Zorenko, 2013), triangle — western Anatolia (*M. h. lydius*), square — southeastern Anatolia (*M. guentheri*). The barrier of isolation is marked with lines: black line — Anatolian Diagonal, white dotted line — Bosphorus Strait, grey line — Maritsa River.



Linear and weight measurements

Brain and olfactory bulbs and cerebellum were weighted (± 0.01 mg) after kept air dry for 5 minutes. Measurements were taken using a digital calliper (± 0.1 mm): length, width and height of the brain; length and width of olfactory bulbs; hemispheres medial and lateral length, length, width and square of pons (pons Varolii) and trapezoid body; optical tubercles and acoustical tubercles of corpora quadrigemina, and finally, width of cerebellum with flocculonodular lobes (Fig. 2). The cephalization index (brain mass square relation to body

Fig. 2. Schematic illustration of vole brain structure and measurements assessed on its anatomical parts in dorsal view (A), ventral view (B) and lateral view (C): 1 — cerebral hemisphere, 2 — hemispheres lateral length, 3 — hemispheres medial length, 4 — rhinal gyrus, 5 — olfactory bulbs, 6 — external olfactory tracts, 7 — olfactory gyrus, 8 — olfactory tubercles, 9 — optic chiasm, 10 — tuber cinereum, 11 — hypothalamus, 12 — cerebral peduncles, 13 — pons varolii, 14 — trapezoid body, 15 — striate parapyramidales, 16 — medulla oblongata, 17 — optical tubercles, 18 — acoustical tubercles of corpora quadrigemina, 19 — flocculonodular lobes, 20 — cerebellum hemisphere, 21 — cerebellum vermis.

mass $\times 100$) was also calculated. In total, 24 parameters were characterized.

The mean value and errors were calculated for all measurements of brain parameters; pairwise comparisons between taxa were performed by using Wilcoxon test (RStudio 3.5.0). We accepted the level of significance at $p < 0.05$.

Microscopy

Brains with optically visible mechanical damage were disclosed from the further manipulations before GM. Brains of 31 vole individual (Tab. 1) were observed in Leica MSV266 stereomicroscope. Pictures were photographed using a digital camera operated by the software Leica Application Suite, ver. 1.06d. Brains were air dried for 5 minutes on the filter paper before photographed to avoid reflection of light occurring in wet regions of the surface. Afterwards, each brain was fixed on the depression (depth — 2 mm) of a movable polystyrene plate (100 mm width/100 mm length/10 mm height). The magnification of 0.65 times was used and appropriate focus was set for each specimen. Equal view and parameters (height, angles of precise dorsal or lateral projecting and the magnification of 65 \times) were used among all brain samples photographed. Taxon, sex, position (lateral or dorsal) and the three-digit code of the individual ID were coded in the name for each picture and file was saved in the tiff format (digital resolution — 2560/1920 pixels).

Geometric morphometrics and shape analysis

The 23 landmarks from the lateral and 22 landmarks from the dorsal side (Fig. 3, Tab. 2) were digitized by tpsDig32 (Rohlf, 2016). Lateral and dorsal projections were used as two different datasets. In the case of the dorsal projection, several landmarks (1, 11, 14, 15, 22) were disclosed from the analysis due to a significant number of missing landmark points among the dataset. Taxa and sex were treated as variables (individuals or factors). MorphoJ (Klingenberg, 2011) was used to perform Generalized Procrustes Analysis (GPA), Principal Component Analysis (PCA), Canonical Variance Analysis (CVA) and Discriminant Function Analysis (DFA).

Results

Size differences

The effect of sex on the length of the brain is not very pronounced. All linear and weight parameters showed no statistic differences ($p > 0.1$). The obtained results allowed combining the females and males data in one sample, characterizing the taxon for brain size.

Three taxa of voles differ significantly in both weight and linear parameters (Tab. 3). Among the studied taxa, the number of statistically significant differences ranged from 16 to 23 (out of 24 studied). The most significant differences were noted between *M. guentheri* compared and *M. hartingi* — by 22–23 parameters. The level of differences between the two subspecies of *M. hartingi* is lower (16 parameters) (Fig. 4).

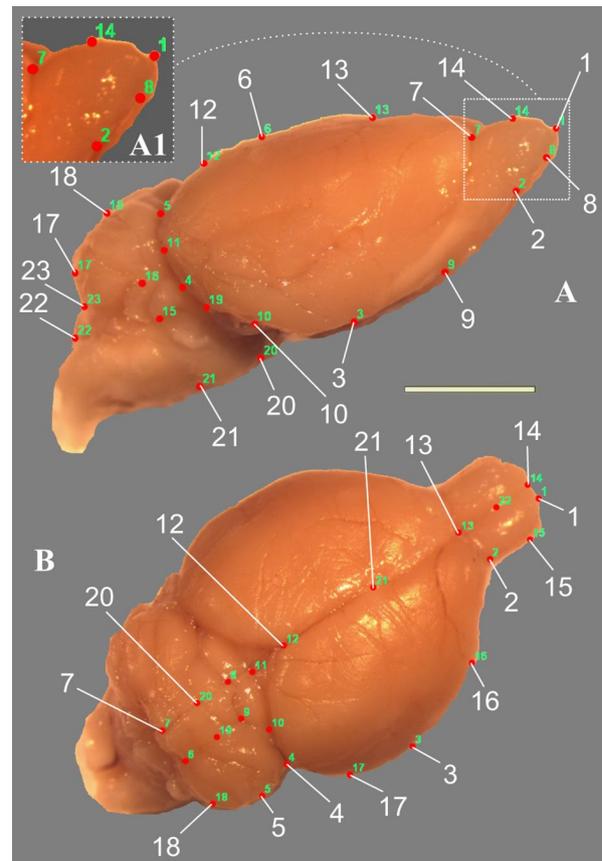


Fig. 3. The landmark locations pointed with red dots (scale bar: 5 mm); A: lateral projection of brain (A1 — magnified part of the brain with landmarks position and symbols size like on a screen of tpsDIG; green numbers indicate landmark numbers), B: dorsal projection of brain. The white numbers correspond to the landmarks.

Table 1. Characteristics of the sample sizes per each dataset used in the statistical analysis, among various taxa of “guentheri” group (number of disclosed brains during statistical analysis of geometric morphometrics are indicated in brackets).

Taxa	Number of brain specimens					
	lateral projection			dorsal projection		
	fe-males	males	total	fe-males	males	total
<i>M. h. hartingi</i>	6 (2)	7 (2)	13 (4)	6 (1)	7 (1)	13 (2)
<i>M. guentheri</i>	3 (1)	3	6	3	3 (2)	6 (2)
<i>M. h. lydius</i>	6	6 (1)	12 (1)	6 (1)	6 (1)	12 (2)
Total	31 (5)			31 (6)		

Shape differences

Procrustes ANOVA test confirmed significant difference in the size (as centroid size) and shape of both dorsal and lateral projections between the three

Table 2. Anatomical and mathematical landmarks used to assess geometric morphometrics of brain of “guentheri” group voles (semi-landmarks or so-called mathematical landmarks indicated with the asterisk *).

Landmark number	Description of landmark location	
	Lateral projection	Dorsal projection
1	Point of terminal tip of olfactory bulbs (apical position)	Point of terminal tip of olfactory bulbs (marginal position)
2	Transition point between olfactory bulbs and external olfactory tracts	Transition point between olfactory bulbs and hemisphere (marginal position)
3	Point of maximum height in cerebral hemisphere main axis (basal position)	Point of maximum width in cerebral hemisphere main axis (marginal position)
4	Point of maximum length of hemisphere main axis (frontal position)	Point of maximum length of hemisphere main axis (marginal position)
5	Transition point between cerebellum and cerebral hemisphere	Point of maximum width of cerebellum main axis (marginal position)
6	Point of maximum height in cerebral hemisphere main axis (apical position)	Transition point between vermis and hemisphere (frontal position)
7	Transition point between olfactory bulbs and cerebral hemisphere (frontal position)	Transition point between left and right cerebellum hemisphere (frontal position)
8	Point at half of the distance between landmarks 1 and 2 *	Transition point between vermis and acoustical tubercles of corpora quadrigemina (medial position)
9	Point at half of the distance between landmarks 2 and 3 *	Transition point between vermis and acoustical tubercles of corpora quadrigemina (marginal position)
10	Point at half of the distance between landmarks 3 and 4 *	Transition point between cerebellum and acoustical tubercles of corpora quadrigemina (apical position)
11	Point at half of the distance between landmarks 4 and 5 *	Transition point between left and right acoustical tubercles of corpora quadrigemina (frontal position)
12	Point at half of the distance between landmarks 5 and 6 *	Transition point between left and right cerebral hemisphere (frontal position)
13	Point at half of the distance between landmarks 6 and 7 *	Transition point between olfactory bulbs and cerebral hemisphere (medial position)
14	Point at half of the distance between landmarks 1 and 7 *	Transition point between left and right olfactory bulbs (caudal position)
15	Point of paraflokuluss attachment to cerebellum (basal position)	Point at half of the distance between landmarks 1 and 2 *
16	Point of paraflokuluss attachment to cerebellum (apical position)	Point at half of the distance between landmarks 2 and 3 *
17	Point of maximum height in cerebellum main axis (frontal position)	Point at half of the distance between landmarks 3 and 4 *
18	Point of maximum height in cerebellum main axis (apical position)	Point at half of the distance between landmarks 5 and 6 *
19	Transition point between cerebellum and medulla	Point at half of the distance between landmarks 6 and 9 *
20	Point of the insertion of trapezoid body (basal position)	Point at half of the distance between landmarks 7 and 8 *
21	Point of terminal tip of trapezoid body (marginal position)	Point at half of the distance between landmarks 12 and 13 *
22	Point of the terminal tip of the visible part of cerebellum vermis (basal position)	Point at half of the distance between landmarks 13 and 14 *
23	Point of the terminal tip of the visible part of cerebellum vermis (apical position)	

taxa. However, there was no significant difference in the shape of the brain between males and females and the centroid size was registered significantly different between males and females only in dorsal projected images of investigated *M. hartingi* and *M. guentheri* vole brains (Tab. 4).

In PCA analysis, the first two PCs explained 46.72 % of morphological variation for data of lateral projection,

from which the PC1 explained 30.42 % of variability. Similarly, data of morphological variation in dorsal projection were explained by 43.79 % variation by first two PCs, from which the PC1 explained 24.23% of data variance. Samples were relatively separately distributed among PC1 and PC2 axes for both lateral (Fig. 5A, left) and dorsal (Fig. 5B, left) projections in the PCA plots. However, the shape difference between the three

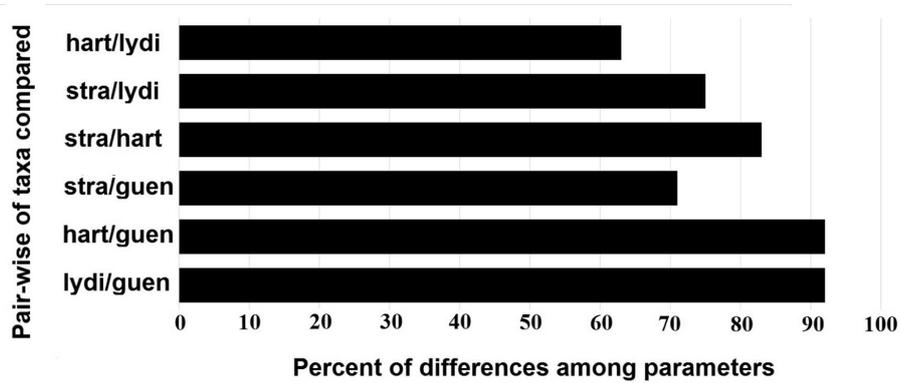


Fig. 4. Differences in linear and weight parameters of brain's structure in percent. Symbols: lydi — *M. h. lydius*, hart — *M. h. hartingi*, stra — *M. h. strandzensis* (Zorenko, 2013), guen — *M. guentheri*.

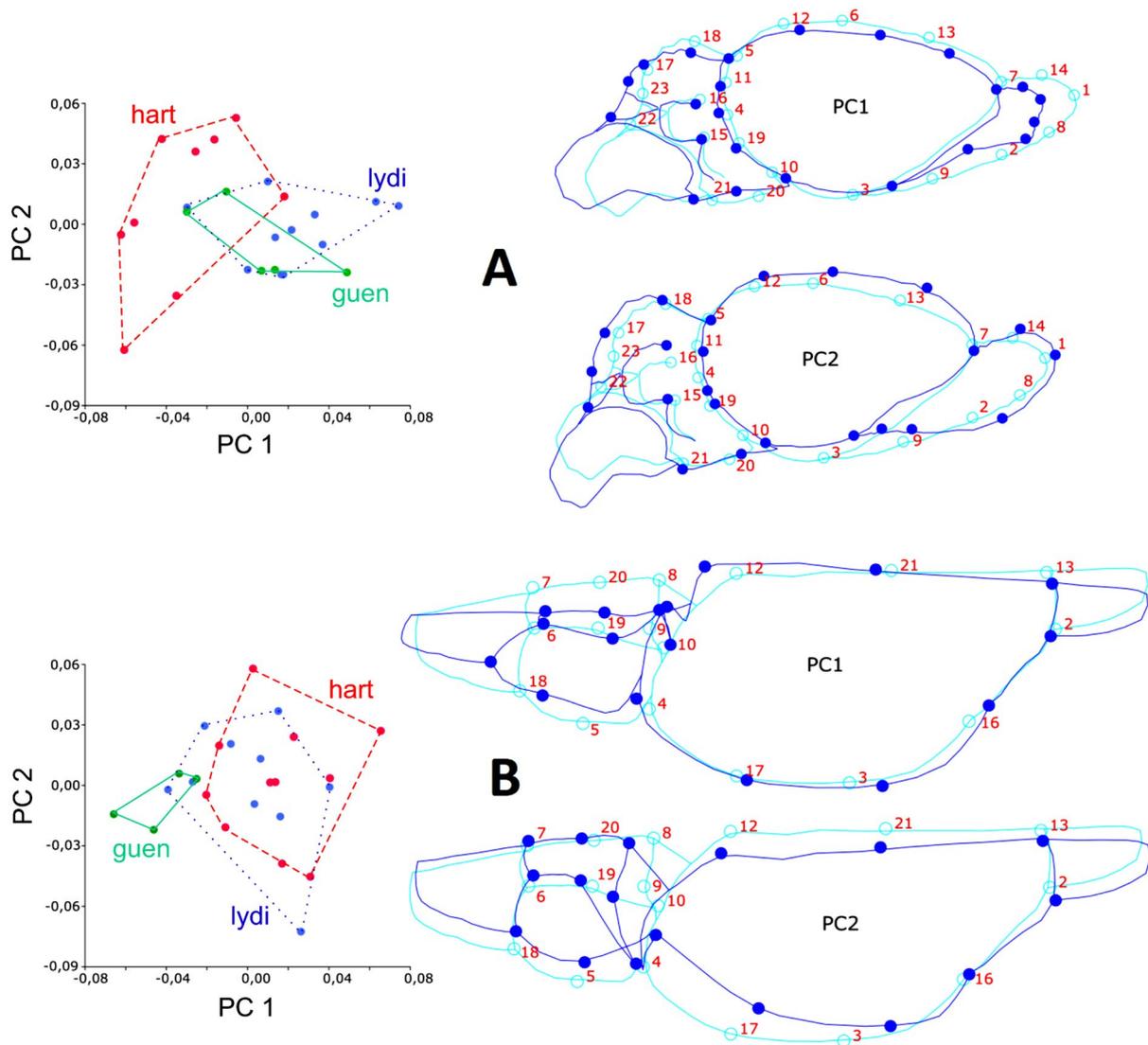


Fig. 5. Scatter plots of PCA (on left) and positive directional shape changes of PC1 and PC2 (on right) of lateral (A) and dorsal (B) projections among of three vole taxa of “guentheri” group (abbreviations see in Fig. 4). Blue dots indicate landmarks after shape deformation; red numbers indicate landmark numbers; scale factor for deformation visualisation — 1.5.

Table 3. The main mean linear and mass parameters of the vole brain.

Parameters	<i>M. h. lydius</i>	<i>M. h. hartingi</i>	<i>M. guentheri</i>
Brain mass, g	0.82±0.01	0.87±0.01	0.57±0.01
Cephalization index	1.3±0.41	1.5±0.51	0.9±0.07
Brain height, mm	7.1±0.10	7.5±0.11	6.5±0.11
Brain length, mm	21.4±0.12	21.3±0.21	19.1±0.33
Brain width, mm	11.6±0.09	11.6±0.11	9.9±0.17
Hemispheres lateral length, mm	11.4±0.07	11.0±0.10	9.6±0.10
Hemispheres medial length, mm	8.4±0.08	8.7±0.08	7.3±0.10
Olfactory bulbs length, mm	3.1±0.08	3.4±0.07	2.7±0.14
Olfactory bulbs width, mm	3.3±0.09	3.7±0.09	3.0±0.12
Olfactory bulbs mass, mg	22.1±1.18	24.9±0.97	14.9±0.67
Cerebellum width with flocculonodular lobes, mm	9.2±0.08	9.2±0.10	8.1±0.23
Cerebellum mass, g	0.11±0.003	0.11±0.002	0.07±0.003
Optical tubercles of corpora quadrigemina, length, mm	2.3±0.05	2.1±0.04	2.0±0.02
Optical tubercles of corpora quadrigemina, width, mm	4.5±0.06	4.2±0.06	3.7±0.07
Optical tubercles of corpora quadrigemina, area, mm ²	10.3±0.25	8.8±0.22	7.4±0.07
Acoustical tubercles of corpora quadrigemina, length, mm	1.7±0.03	1.6±0.03	1.2±0.02
Acoustical tubercles of corpora quadrigemina, width, mm	5.2±0.06	4.6±0.06	4.2±0.13
Acoustical tubercles of corpora quadrigemina, area, mm ²	8.6±0.21	7.5±0.17	5.2±0.20
Trapezoid body length, mm	1.7±0.03	1.6±0.04	1.5±0.08
Trapezoid body width, mm	4.9±0.06	5.0±0.06	4.1±0.09
Trapezoid body area, mm ²	8.2±0.15	7.9±0.22	6.1±0.36
Pons length, mm	1.6±0.02	1.7±0.04	1.4±0.06
Pons width, mm	3.8±0.07	3.9±0.10	3.2±0.09
Pons area, mm ²	5.9±0.13	6.6±0.23	4.7±0.20

Table 4. Procrustes ANOVA results (F — Goodal's F, CS — centroid size, significant difference marked in bold).

Individuals	Dataset		F	<i>p</i> -value	Individuals	Dataset		F	<i>p</i> -value
Taxa	Dorsal	CS	40.99	<0.0001	Sex	Dorsal	CS	5.41	0.0292
		Shape	2.11	<0.0001			Shape	1.21	0.2087
	Lateral	CS	38.38	<0.0001		Lateral	CS	1.46	0.2394
		Shape	2.75	<0.0001			Shape	0.82	0.7891

“guentheri” group taxa (Fig. 5A and B, right) was similar to that calculated by CVA plots and is explained in the further text.

Samples of all taxa significantly differed in shape for both lateral and dorsal projection according to permutation *p* values based on Mahalanobis distance in CVA. According to permutation *p* values based on Procrustes distance, significant difference in shape was registered only between taxa of *M. guentheri* and *M. h. hartingi* and between taxa of *M. guentheri* and

M. h. lydius for dorsal projections (Tab. 5) as well as only between taxa of *M. h. lydius* and *M. h. hartingi* for lateral projections (Tab. 5).

Results of DFA analysis also showed no statistically significant difference in shape between males and females for both lateral and dorsal projections (Tab. 6). However, during DFA several testing values proved that in both lateral and dorsal projections the shape deformation is significantly high among compared vole taxa (Tab. 7).

Shape differences (deformation) between the mentioned taxa were also well separated along first axis of the scatter plot of CVA (Fig. 6). In CVA, the CV1 for dorsal projection explain 74.62%, and CV1 for lateral projection — 76.40% of the total variation of shape among the three taxa. Most significant shape changes of the positive and negative directional deformation according to CV1 were the prolongation of olfactory bulbs, thickening of flocculonodular lobes and thickening of cerebral hemispheres for lateral projections (Fig. 6A)

as well as the thickening of cerebellum hemisphere for dorsal projections (Fig. 6B) between the taxa *M. guentheri* and *M. h. hartingi*. Olfactory bulbs in samples of *M. h. hartingi* were more elongated anteriorly compared to *M. h. guentheri* that had shorter olfactory bulbs (Fig. 6A). Likewise, shortening of olfactory bulbs and prolongation of cerebral hemispheres for lateral projections between the taxa *M. h. hartingi* and *M. h. lydius* were the most significant shape changes according to CV1 (Fig. 6C).

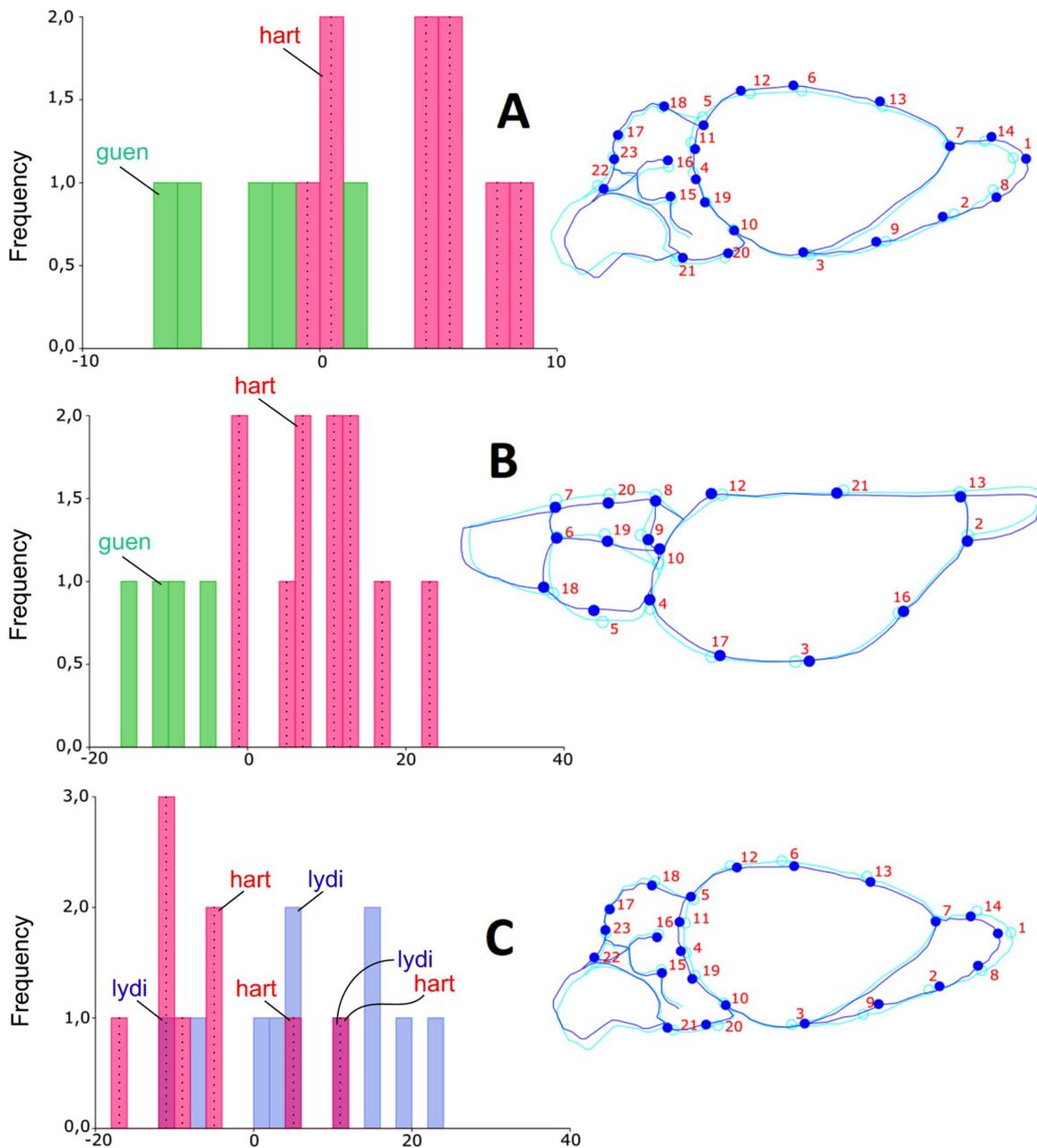


Fig. 6. DFA histogram (left) and CVA shape deformation results for lateral (A, C) and dorsal (B) projections (right) of three vole taxa of “guentheri” group (abbreviations see in Fig. 4). Blue dots indicate landmarks after shape deformation; red numbers indicate landmark numbers; scale factor for deformation visualisation — 1.0.

Table 5. CVA results for lateral and dorsal projections (Mah. Dist. — Mahalanobis distance; Proc. Dist. — Procrustes distance; Perm. p — permutation p value; bold — statistically significant difference).

Groups	<i>M. guentheri</i>				<i>M. h. hartingi</i>			
	Mah.dist.	Perm.p.	Proc.dist.	Perm.p.	Mah.dist.	Perm.p.	Proc.dist.	Perm.p.
Lateral projection								
<i>M. h. hartingi</i>	4.1625	0.0022	0.0516	0.0539	–	–	–	–
<i>M. h. lydius</i>	4.7956	0.0004	0.0360	0.1308	5.5188	<0.0001	0.0592	0.0005
Dorsal projection								
<i>M. h. hartingi</i>	7.7251	<0.0001	0.0625	0.0012	–	–	–	–
<i>M. h. lydius</i>	6.8804	0.0001	0.0511	0.0205	3.5722	<0.0001	0.0301	0.2346

Table 6. DFA results for lateral and dorsal projections among vole sexes (T2 — T-square; Param. p — parametric p values; Perm. p — permutation p value; Proc. — Procrustes distance value; bold — significant difference).

	Females			
	T2	Param.p.	Perm.p. (T2)	Perm.p. (Proc.)
Lateral projection				
Males	41.0745	0.9984	0.5700	0.5910
Dorsal projection				
Males	78.0953	0.9841	0.7190	0.2490

Table 7. DFA results for lateral and dorsal projections among different vole taxa (T2 — T-square; Param. p — parametric p values; Perm. p — permutation p value; Proc. — Procrustes distance value; bold — significant difference).

Groups	<i>M. guentheri</i>				<i>M. h. hartingi</i>			
	T2	Param.p.	Perm.p. (T2)	Perm.p. (Proc.)	T2	Param.p.	Perm.p. (T2)	Perm.p. (Proc.)
Lateral projection								
<i>M. h. hartingi</i>	41.6041	0.9125	0.0150	0.0430	–	–	–	–
<i>M. h. lydius</i>	33.9276	0.9693	0.0490	0.1350	108.5430	0.8988	0.0080	<0.0001
Dorsal projection								
<i>M. h. hartingi</i>	91.2031	0.8034	0.0010	0.0020	–	–	–	–
<i>M. h. lydius</i>	35.8342	0.9319	0.0900	0.0290	54.8047	0.9811	0.2730	0.2300

Discussion

In order to use morphometry, especially the geometric method, it is important to exclude the possible impact of age and gender on certain structures (Barčiová, 2009). The results of the linear morphometry show that sex dimorphism of voles is not pronounced. The male and female voles studied did not differ significantly with regard to the dorsal and ventral side of the brain in linear and geometric morphometry (Tabs 7 and 8). Apparently, sexual selection does not affect the parameters of the brain. This pattern has been demonstrated in many vole species of the Microtini tribe (Zorenko, 2013).

Age variability has a great influence on the size and shape of the morphological structure (Barčiová, 2009; Voyta *et al.*, 2013; Orbach *et al.*, 2017). The vole's brain development finishes early in comparison with the growth of other organs (for example the skull), which reflects the general pattern of ontogenesis in mammals. The absolute and relative mass of the vole's brain increases rapidly especially in the age of 12 days to 20 days when intense brain maturing occurs. At this age, the vole's brain is ready to perceive the most important signals of the external environment and is able to ensure the formation of basic patterns of behaviour. Starting from the 20th day, brain growth slows down; its relative

weight begins to decrease. Two months old voles do not differ from adults in the structure of the brain, as well as in the relative mass of the brain, which indicates the completion of its development. However, the absolute mass is still increasing. Thus, the allometric factor is very important. Therefore, for morphometric analysis, adult animals were taken, in which the growth of the body was complete.

During geometric morphometric analysis, we detected several shape differences between the three “guentheri” taxa (Tabs. 4, 5 and 7; see also Figs. 5 and 6). The prolongation of olfactory bulbs, thickening of flocculonodular lobes and thickening of cerebral hemispheres for lateral projections (Fig. 6A) as well as the thickening of cerebellum hemisphere for dorsal projections (Fig. 6B) were the most significant shape deformations between the taxa *M. guentheri* and *M. h. hartingi* (see also PC1 in Fig. 5A). Olfactory bulbs in samples of *M. h. hartingi* were more elongated anteriorly compared to *M. guentheri* that had shorter olfactory bulbs (Fig. 6A). Likewise, shortening of olfactory bulbs and prolongation of cerebral hemispheres for lateral projections between the taxa *M. h. hartingi* and *M. h. lydius* were the most significant shape changes according to PC1 (Fig. 5A) and CV1 (Fig. 6C).

Nevertheless, the geometric morphometric analysis was executed with relatively small sample sizes for each data sub-group (2, 3, 6–13) which can conduct to the problem of low diversity of investigated individuals. In most cases that is not enough to characterize morphological diversity in the referenced taxa, yet commonly is seen in other similar published studies (Voyta *et al.*, 2013; Kamilari *et al.*, 2013; Orbach *et al.*, 2017; Kaya *et al.*, 2018).

All studied taxa differ noticeably in most linear parameters of the brain — the level of differences varies from 67 to 96% (Fig. 4). In *M. guentheri*, most of the parameters are not only different, but almost do not overlap with those of *M. hartingi*. A large number of brain signs overlap between the subspecies of *M. hartingi*, so the level of differences is reduced to 67% (*M. h. hartingi*/*M. h. lydius*) and 75% (*M. h. strandzensis*/*M. h. lydius*) (see also Fig. 4). An exception is the *M. h. hartingi* subspecies, whose brain is heavier, longer and wider; the differences are noted in the mass and length of the bulbs, so the level of differences is already equal to 83%.

We predicted that geographical location would influence the vole brain. Linear and geometric morphometric data indicate that individuals from different populations (especially for the three subspecies of *M. hartingi*) differ significantly (Fig. 4, Tab. 3), which may depend on local ecological conditions, and they were supported by selection. Variability in brain size has been demonstrated in subspecies of *Microtus socialis* Pallas, 1779 (Zorenko, 2013). Perhaps a change in geometric patterns affects the value of the indices of the brain. A similar hypothesis for the sections of the brain cortex of *Microtus arvalis* Pallas, 1779 was expressed (Yaskin & Lenec, 1996). Brain geometry is influenced by the volumetric proportions of its components, as well as by

their spatial organization and patterns of connections (Bruner *et al.*, 2014). Shape differences can be the result of changes in the relative position of neural elements of the brain and may have played a particularly relevant role in human brain (Bruner, 2004).

The vole brain size is highly variable within a species. Variability was shown in many mammal species too (Kovalenko, 1982; Khrustaleva *et al.*, 1994; Zorenko, 2013). The analysis of brain size parameters provided the opportunity to prepare key tables to distinguish between genera and species of voles (Zorenko, 2013). Geometric morphometric analysis showed significant differences in the brain shape of voles as well. Therefore, we can talk about the important role of a phylogenetic signal.

Functional morphology may have a definite meaning. Thus, the dimensions of the optical tubercles and acoustical tubercles are associated with the degree of development of vision and hearing. In animals with more advanced vision, optical tubercles dominate; in more developed hearing, acoustical tubercles are dominated (Khrustaleva *et al.*, 1994). However, this is a simplified view of the functions of the corpora quadrigemina. Optical tubercles play an important role in ensuring systemic reactions (eye movements, earlap, heads and vibrissae) and, therefore, they are one of the elements of integrative brain systems of mammals. An approximate reaction to the sound signal with the participation of the acoustical tubercles occurs (Andreeva & Obukhov, 1999). The size of the olfactory bulbs depends on the nature of the food: in seed-eating species, they are larger than in green-eating ones.

As noted in the introduction, more recently, Gunther’s vole, which has a large range, was considered as a single species (Musser & Carleton, 2005; Kryštufek & Vohralik, 2005). However, molecular (Kryštufek *et al.*, 2009) and morphological (Yiğit & Çolak, 2002; Yiğit *et al.*, 2012; Markov *et al.*, 2014; Zorenko & Golenishchev, 2015) studies found evidences showed significant differences in all investigated populations, which could be a result of isolation of populations during the resettlement of voles from the centre of origin and the subsequent formation of the range.

Molecular evidence indicates that southwest Asia was the cradle for voles of the subgenus *Sumeriomys* Argyropulo, 1993 (Kryštufek *et al.*, 2009), and this group was numerous in the fossils from the Early Pleistocene (Goren-Inbar *et al.*, 2000) and later (Marder *et al.*, 2011). In the same region, the formation of vole group “guentheri” took place (Yiğit *et al.*, 2017). Prolonged isolation by the mountain Anatolian Diagonal of a part of the ancient population, which is close to *M. h. lydius*, contributed to the accumulation of differences, including morphological ones.

M. guentheri statistically reliably differs from *M. hartingi* in absolute mass of the brain and its separate parts (olfactory bulbs, cerebellum, and large hemispheres), as well as in most linear parameters of the brain (Fig. 4, Tab. 3). The brain of this taxon also differs in its shape (both frontally and laterally). The differences in linear and weight parameters of *M. guentheri* are

explained by smaller brain sizes, while body size has little effect on brain size: the correlation of brain mass and body is only 0.09; brain length and body length 0.48 ($p < 0.001$). Significant differences in the brain compared with the three taxa of *M. hartingi* were noted for 22–23 indicators (Fig. 4).

The resettlement of voles to Europe and the subsequent isolation of new European populations from the maternal population of the Bosphorus Strait led to their divergence. The formation of the *M. hartingi* range in Europe apparently occurred under conditions of fragmented habitats, which was determined by the influence of climate fluctuations in the Late Pleistocene and Holocene. Currently, separate fragments with different size (Kryštufek *et al.*, 2018) represent the range. Fragmentation of friendly habitats and isolation from the original population could contribute to morphological divergence. Harting's vole probably colonized Europe (Kryštufek *et al.*, 2018) from Anatolia through a land corridor that allowed fauna exchange between the regions from the Middle Pleistocene (McHugh *et al.*, 2008). Finally, 7–5.3 thousand years ago the corridor was flooded with a significant rise in sea level (Kerey *et al.*, 2004).

The emergence of the Bosphorus Strait and the separation of part of the population in southern Europe also contributed to the morphological differences. The subspecies *M. h. hartingi* has the largest brain and olfactory bulbs. Brain length and width in *M. h. hartingi* and *M. h. lydius* are similar; however, the height is significantly greater. The ratio of the parameters of the hemispheres of this subspecies is changed: a large medial and a shorter lateral length. In total, there are sixteen differences in parameters out of 24 (67%) (Fig. 4).

Another subspecies *M. h. strandzensis*, whose brain sizes also have significant differences, inhabits the southeastern part of the Balkans (Zorenko, 2013). *M. h. strandzensis* differs from *M. h. lydius* by 18 parameters (75%), whereas from *M. h. hartingi* by 20 (83%). It has been established that *M. h. strandzensis* olfactory bulbs are long and relatively narrow, whereas in the other two subspecies they are almost equal in both length and width. The cerebellum has the lowest mass (92.8 mg, i.e., 12.5 mg less than *M. h. lydius* and *M. h. hartingi*). In this taxon, the optical tubercles and acoustical tubercles have almost the same square, whereas in *M. h. lydius* the square of the optical tubercles is larger than the acoustical but in *M. h. hartingi* smaller.

The results obtained can be explained by the fact that the formation of the range took place over a long period and the degree of isolation between the three vole taxa (*M. h. lydius*, *M. h. strandzensis* and *M. h. hartingi*) is not the same. There is evidence that the Harting's vole settled Greece (Boeotia) from the Late Pleistocene to the Early Neolithic (Wilczyński *et al.*, 2016). Perhaps the Rhodope vole population is connected with the Greek populations, forming one of the large fragments of the current range. It is possible that these are the most ancient populations. Therefore, *M. h. hartingi* is distinguished by a smaller divergence in the structure of the brain, as compared to *M. h. lydius*. In the region of the southeastern Balkans

repeated colonization from Asia was possible, which affected less pronounced morphological differences between *M. h. strandzensis* and *M. h. lydius* (Thanou *et al.*, 2012; Kryštufek *et al.*, 2018).

In conclusion, it should be noted that *M. guentheri* is clearly isolated from the *M. hartingi* in size and shape of the brain, which is consistent with the data obtained on the structure of the skull, bacula, and spermatozoa, as well as the molecular characteristics of the species. Despite the similarity of molecular characteristics, the Harting's vole is not so uniform morphologically, which indicates an active process of morphological evolution and divergence in the Harting's vole. *M. h. lydius* is clearly isolated, and noticeable differences are found between *M. h. hartingi* and *M. h. strandzensis*. Evolutionary transformations of the shape of the brain could cause such significant changes in its linear parameters.

A high morphological divergence and accelerated evolutionary changes were noted for the *M. hartingi* of the fragmentation of habitats (Kryštufek *et al.*, 2018). Morphological changes in response to fragmentation are similar to changes in island species (Millien, 2006, 2011). It has been shown that body size changes in 25 Danish mammals over the past two centuries have followed the island rule and were attributed to habitat fragmentation (Schmidt & Jensen, 2003). The same factors as on the islands apparently were acting in the small fragmented populations of the Harting's vole in Europe. The main factor could be a decrease in habitat area, while the absence of predators, a decrease in interspecific competition and limitation of resources (Dayan & Simberloff, 1998; Guthrie, 2003) apparently, did not act. Morphological divergence often occurs in relatively short periods, whereas molecular changes are more often associated with long periods. Therefore, the absence of correspondence between morphological and molecular evolution is possible.

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