

Variation in shell morphology of the European pond turtle, *Emys orbicularis*, in fragmented central European populations

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The European pond turtle (*Emys orbicularis*) is a widely distributed freshwater species inhabiting much of Europe, but it is often in population decrease or is locally extinct. In this study, we sampled five central European populations, of which four were autochthonous and one was introduced outside the native range. Moreover, two of the native populations were relatively isolated and at the periphery of the species range. Using the frequency of shell anomalies, a geometric morphometric framework and an analysis of fluctuating asymmetry, we aimed to determine the degree of morphological differentiation among different populations. Significantly, a smaller number of individuals with a malformed shell or scutes occurred in the native core range population, which potentially has a high level of gene flow (Hungary). Although neither canonical variate analysis nor the morphological disparity analysis showed distinct differences between populations, we found significantly higher shell asymmetry in the two marginal populations (Austria and Slovakia) compared with the core range populations. Our results might thus support the central–marginal hypothesis and indicate potential genetically based conservation problems owing to demographic bottlenecks and/or isolation in marginal populations.

ADDITIONAL KEYWORDS: fluctuating asymmetry – geometric morphometrics – isolated populations – population history – tortoise.

INTRODUCTION

Freshwater turtles belong to the most endangered group of vertebrates, and they are often threatened with extinction or extirpation of local populations (Markovic *et al.*, 2014; Rhodin *et al.*, 2018). The most significant threats to turtle biodiversity are habitat loss and fragmentation, followed by unsustainable collection for consumption and the pet trade, unrestrained (re-)introductions, pollution and climate change (Stanford *et al.*, 2020). Furthermore, biological

invasions, changes in population demography and life-history parameters have all been reported to affect the risk of extinction of various turtle species considerably (e.g. Araújo *et al.*, 2006; Nori *et al.*, 2017; Cortés-Gómez *et al.*, 2018).

In freshwater turtles, owing to the complexity of their shell structure (Pritchard, 2007), the impacts of suboptimal environmental conditions and genetic abnormalities can be detected with relative ease by tracking expressions in phenotypic variability or developmental instability, represented by changes in shell shape, asymmetry or various scale malformations, additions or reductions (e.g.

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Zangerl & Johnson, 1957; Velo-Antón *et al.*, 2011; Goessling *et al.*, 2017). There is good evidence that these shell abnormalities are induced by temperature and humidity constraints during incubation, by environmental pollution or by loss of genetic diversity in bottlenecked or inbred populations (Ayres Fernández & Cordero Rivera, 2004; Velo-Antón *et al.*, 2011; Nagle *et al.*, 2018). For example, high temperatures during embryonic development in natural nests can induce scale abnormalities, leading to the formation of additional and malformed scutes in turtle hatchlings, which can subsequently lead to a reduction of fitness of entire cohorts (Telemeco *et al.*, 2013). Furthermore, an abnormal shell shape could heavily impact reproductive performance traits of turtles and, in some cases, these changes could even affect reproductive output in different habitat conditions (Bell *et al.*, 2006; Rivera, 2008).

Environmental and genetic stress, leading to developmental instabilities, can also increase levels of fluctuating asymmetry (FA), which refers to stochastic deviations from perfect symmetry in bilaterally symmetric organisms (Dongen, 2006; Graham *et al.*, 2010). Fluctuating asymmetry is recognized in several reptile species as an indicator of environmental stress that could weaken individual fitness (Herczeg *et al.*, 2005; Ljubisavljević *et al.*, 2005; Lazić *et al.*, 2013). Additionally, deviations from perfect symmetry could also be caused by loss of genetic variation (Garrido & Pérez-Mellado, 2014), by population isolation (Băncilă *et al.*, 2010) and by habitat fragmentation (Sarre, 1996). For example, Goessling *et al.* (2017) found higher FA in populations of gopher tortoises (*Gopherus polyphemus*) with low genetic diversity. Patterns of developmental instability in plastron shape were also detected in a Eurasian spur-thighed tortoise (*Testudo graeca iberica*) population (Băncilă *et al.*, 2012).

The occurrence of carapace anomalies in the European pond turtle (*Emys orbicularis*) was reported in different parts of its distribution range (Balázs & Györffy, 2006; Najbar & Szuszkiewicz, 2006), and, furthermore, the degree of population isolation was believed to be one of the factors shaping observed morphological variation (Cordero Rivera *et al.*, 2008; Velo-Antón *et al.*, 2011). These findings are also supported by the central–marginal hypothesis, which posits that isolated and peripheral populations occurring in marginal habitats have lower genetic diversity and greater genetic differentiation compared with central populations, which can, consequently, affect their fitness (Eckert *et al.*, 2008; Gassert *et al.*, 2013; Langin *et al.*, 2017). In contrast to the centre of the species range, limited gene flow among neighbouring populations at the range margin makes them more prone to severe demographic bottleneck (Schwartz *et al.*, 2003; Vandewoestijne *et al.*, 2008).

The historical range of the European pond turtle covered considerable parts of the Western Palaearctic; however, owing to various factors (habitat loss, dispersal barriers, human disturbance, climate change, etc.) it has become discontinuous and heavily fragmented, and the species has disappeared from many areas (Sommer *et al.*, 2007, 2009; Pedall *et al.*, 2011; Fritz, 2012). In the early Holocene, favourable climatic and environmental changes enabled the expansion of *E. orbicularis* towards northern Europe; it reached the Pannonian Basin first (Sommer *et al.*, 2007). Accordingly, the biggest continuous distribution area in central Europe is located in Hungary, but the sizes of local populations are mostly decreasing and, despite the lack of comprehensive information regarding their present status, they are considered endangered, mostly owing to habitat fragmentation and gradual isolation of native populations (Puky *et al.*, 2004; Schindler, 2008; Farkas *et al.*, 2013; Jablonski *et al.*, 2015). Thus, the fragmented distribution range of *E. orbicularis* in central Europe today is an outcome of more consecutive range fluctuations, resulting in genetic differentiation of more or less isolated populations (Fritz *et al.*, 2007). Current changes in phenotypic traits caused by suboptimal environmental conditions or reduced gene flow in peripheral areas of the species range are thus expected.

In the present study, using the frequency of anomalies, a geometric morphometric framework and the fluctuating asymmetry of the shell, we aimed to determine the degree of morphological differentiation among different populations of *E. orbicularis* in central Europe. For our analysis, we used populations from Hungary, Slovakia, Austria and Czechia, which have different population histories and, presumably, different levels of gene flow. We expected higher levels of morphological differentiation and higher FA scores caused by possible genetic drift owing to limited gene flow in marginal native populations in Austria and Slovakia and in an isolated introduced Czech population than in those from the Hungarian part of the species range. Owing to the known demographic bottleneck of the Czech population, we also expected an increased incidence of shape deviations there, because such anomalies are known to be more common in introduced populations of other turtle species (Nagle *et al.*, 2018).

All selected native populations belong to mitochondrial lineage II (*E. o. orbicularis*), whereas the introduced Czech population belongs to mitochondrial lineage I (Fritz *et al.*, 2007). Different pond turtle populations not only differ in size and coloration, but the subspecies are characterized by different shell shape, scute shape and relative head size, and also by length ratios of some ventral scutes (Rogner, 2009; Joos *et al.*, 2017). Therefore, one may also hypothesize about phylogenetic origin as

the primary factor explaining potential morphological variation. For this reason, we measured morphological disparity between different lineages.

MATERIAL AND METHODS

STUDY POPULATIONS

Individuals were sampled from four native, autochthonous populations situated in central Hungary (HU), southeastern Hungary (HUs), eastern Slovakia (SK) and eastern Austria (AT) and from one introduced population in southern Moravia (Czechia; CZ; Fig. 1). All populations inhabit more-or-less similar lowland habitats in human-transformed landscapes (elevation range 74–153 m) represented by river branches, oxbows and artificial canals surrounded by woodlands and agricultural lands. The banks of the sampled waterbodies are densely covered by vegetation used by the turtles for basking. Most of the terrestrial habitats are characterized by sandy soils with xerophilous flora. The presence of suitable terrestrial habitats is important for terrestrial activities of the turtles, such as egg-laying and dispersal (Ficetola *et al.*, 2004; Rogner, 2009). Except for the single site of the introduced CZ population, native populations were sampled at three to eight sites, with the average distance between two

adjacent sites being 5–10 km, rarely ≤ 70 km (Fig. 1), where continuous gene flow among them is expected.

Central Hungary (HU)

The species is widely distributed in Hungary, but its range is somewhat discontinuous, mainly owing to river controls introduced in the 19th century (Farkas, 2000; Puky *et al.*, 2004; Farkas *et al.*, 2013). Our sample population inhabits the Sárvíz Valley, i.e. the catchment of the Séd-Nádor Channel, which extends from the confluence of the Nádor Channel and Gaja Stream to the Sió River at Sióagárd in western Hungary. The fish ponds along this river today are relatively modest remnants of a formerly widespread lake system (Babai, 2010). As a whole, the local turtle population appears to be stable, with an appropriate age structure and a constant rate of population growth (B. Farkas & B. Halpern, unpubl. data). The connectivity among these waterbodies suggests continuous gene flow between the populations, which are considered as a single unit in this study.

Southeastern Hungary (HUs)

Owing to its distinct geographical location, the population of Lake Fehér in the Tisza River catchment in southern Hungary was analysed separately from

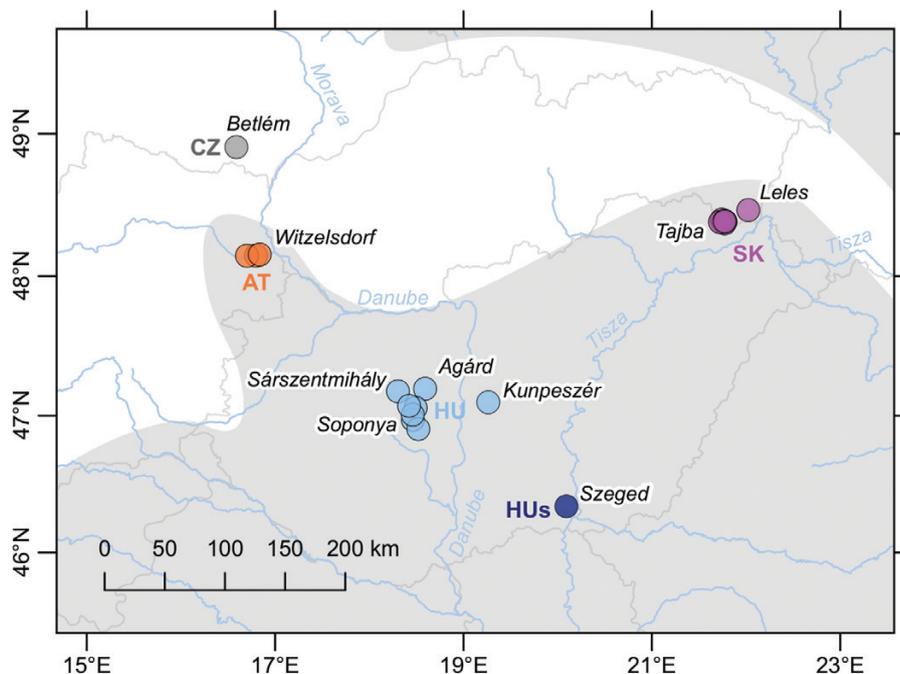


Figure 1. Sampling sites of *Emys orbicularis* in four native populations, in central Hungary (HU), southeastern Hungary (HUs), eastern Slovakia (SK) and eastern Austria (AT), and in one introduced population, in southern Moravia (Czechia; CZ) in central Europe. Labels for site names are limited for better clarity. Shaded area indicates the species range of native populations (adapted from Vamberger & Fritz, 2018).

the other Hungarian samples (HU), which belong to the catchment of the Danube River. Until the early 20th century, Lake Fehér was one of the biggest alkaline lakes in Hungary. Today, it is an extended system of artificial fish ponds connected to the Tisza River, which supports a thriving population of European pond turtles. Lake Fehér fulfils all the most important ecological needs of the species; carcasses of dead fish ensure an important food source, and embankments of fish ponds serve as a suitable egg-laying site for the turtles (B. Csibrány, unpubl. data).

Eastern Slovakia (SK)

The population in eastern Slovakia (Tajba National Nature Reserve) has been a subject of long-term study (e.g. Novotný *et al.*, 2008; Havaš & Danko, 2009; Horváth *et al.*, 2017) and seems to have been isolated for a rather long time, although occasional gene flow thanks to migrating male individuals from adjacent populations in northern Hungary could be expected (P. Havaš, unpubl. data). The Tajba marsh is a 2-km-long and 100- to 150-m-wide oxbow of the river Bodrog. The oxbow is divided into two connected parts; the permanent open water surface is confined to the western part of the oxbow, with a maximum depth of 70 cm (Novotný *et al.*, 2008).

Eastern Austria (AT)

The autochthony of Austrian populations was repeatedly called into question because of the extensive trade in turtles as Lenten food and as pets in the last century (Lutschinger, 1989; Kunst & Gemel, 2000). Currently, the only native *E. orbicularis* population is restricted to a small floodplain area in the Donau-Auen National Park, along the banks of the river Danube. The population we sampled in this study inhabits oxbows and side-arms of the river Danube along a flood-protection dike traversing the floodplain. Despite this restraint, a successfully implemented conservation project on nest predation has allowed the population to increase (Schindler *et al.*, 2017).

Czechia (CZ)

Compared with the other populations described, the situation in Czechia is different, where no surviving native populations occur (Široký *et al.*, 2004; Široký & Moravec, 2015a, b). Therefore, a reintroduction programme took place in 1989–1994, when 30 individuals were imported from the Danube Delta in Romania and released into wetlands in Betlém (south Moravia), in the former inundation area of the River Dyje. The wetland system consists of a main

wetland, with 15 satellite pools with various depth (maximum 1.2 m), spread over an area of 4.5 ha. Currently, the size of this non-native and isolated population is estimated at 247 individuals, and regular reproduction takes place at the site (Šebela, 2015, 2017).

DATA

In the period from 1998 to 2017, a set of 495 carapace and 436 plastron photographs of *E. orbicularis* individuals was collected. All the photographed specimens were catalogued and identified as individuals based on unique identification marks and were released back to the site where they were caught. All individuals were older than 4–5 years (age was estimated according to the number of growth rings on scutes), but we were not able to determine the accurate age structure of studied populations. Initially, this dataset was used to evaluate shell anomalies. Analyses of geometric morphometrics were performed using a subset of photographs of specimens of known sex, i.e. 246 carapaces (154 females and 92 males) and 351 plastrons (207 females and 144 males; Table 1). Raw pictures of shells are stored by the authors and are available upon request to the corresponding author.

EVALUATION OF SHELL ANOMALIES

The normal configuration of scutes in the *E. orbicularis* carapace consists of five vertebral scutes, four pairs of costal scutes, a single nuchal scute and 12 marginal scutes on each side, whereas the plastron is made up of six pairs of scutes (Fig. 2; Rogner, 2009). Possible deviations from the normal arrangement of the carapace and plastron scutes were evaluated in all collected photographs. Any additional or deformed scutes (deviation from the normal curvature of the scute, i.e. elongated or shortened scutes) on the carapace or plastron and any deformation of the shell shape (deviation from the normal curvature of the shell) were considered as anomalous and categorized as accessory scute, abnormal shell shape and abnormal scute shape. Thus, including the normal shell, we had four types of shells according to the possible anomalies. We used Pearson's χ^2 tests to evaluate whether the proportions of anomalies differed significantly among different study populations. To test whether the proportion of anomalies differed among populations we fitted a model of independence for a two-way table. The result was visualized in a mosaic plot using the package 'vcd' v.1.4-6 (Meyer *et al.*, 2020) in the R v.3.6.3 environment for statistical computing (R Core Team, 2020).

Table 1. Number of individuals used for the analyses of morphological variation from each sampling site

Population	Shell anomalies		Analyses of geometric morphometrics			
	Carapace	Plastron	Carapace		Plastron	
			Females	Males	Females	Males
HU	77	82	29	44	33	45
HUs	102	112	27	16	27	18
SK	81	93	57	22	61	29
AT	52	52	41	10	41	10
CZ	183	97	n/a	n/a	45	42

Sampling sites of *Emys orbicularis* were as follows: AT, eastern Austria; CZ, Czechia; HU, central Hungary; HUs, southeastern Hungary; SK eastern Slovakia. Abbreviation: n/a, not available.

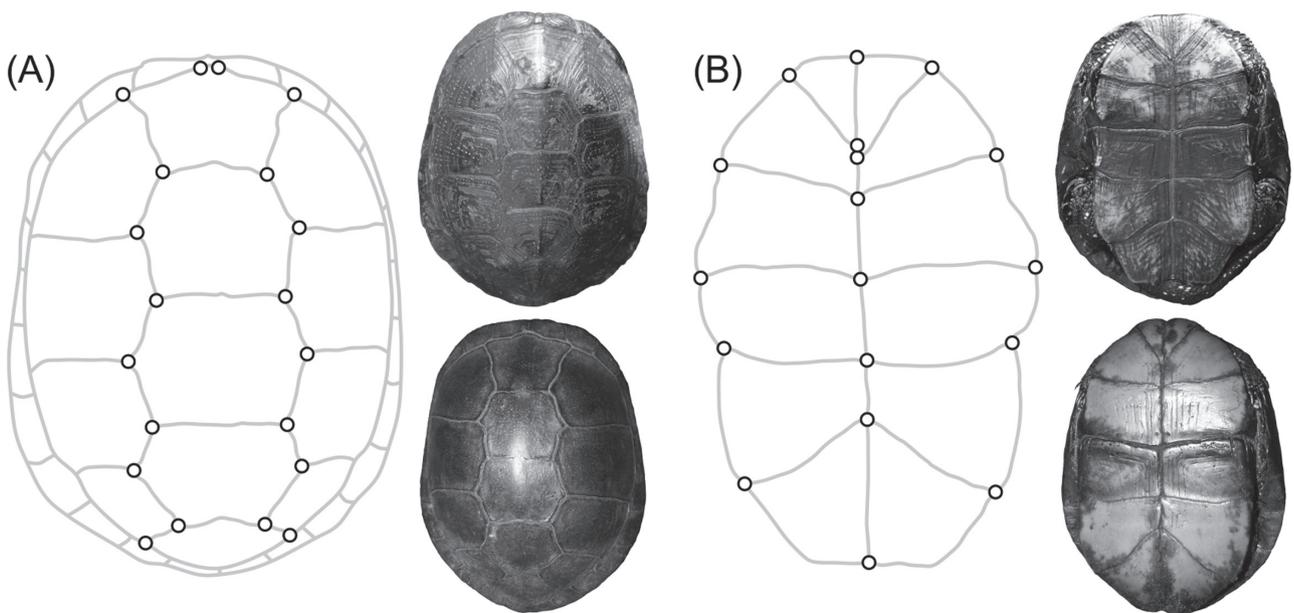


Figure 2. Position of landmarks (circles) used for geometric morphometric analysis of the shell of *Emys orbicularis* in (A) dorsal (carapace) and (B) ventral (plastron) aspect, with cephalic region oriented towards the top. Enclosed photographs depict variation of shell structures.

GEOMETRIC MORPHOMETRIC ANALYSES

Given that plastron and carapace photographs were obtained in the field in different conditions and in different ways, they were adjusted digitally. We did not consider the size, and therefore each photograph was trimmed to the shell edge in the craniocaudal axis and rescaled to 1000 pixels in length. Moreover, all of the pictures were oriented to the same position regarding craniocaudal axis and fitted to the centre. A total of 38 geometric landmarks were identified, 20 on the carapace and 18 on the plastron (Fig. 2). We were not able to add more landmarks on the carapace, because they were not visible on the most of the photographs.

These landmarks corresponded to the intersections between epidermal scutes, which provide highly homologous and reliable characters. However, for two-dimensional geometric morphometrics we were able to digitize only a limited set of carapace landmarks owing to the distinct curvature of the shell (Fig. 2A). Landmark coordinates were acquired from photographs using TPSDIG232 software (Rohlf, 2015) and superimposed following the Procrustes method of generalized least squares superimposition implemented in MORPHOJ software (Klingenberg, 2011) to eliminate non-shape variation (differences in the location or rotation). The shape morphometrics were obtained by elimination

of the centroid size; thus, the resulting superimposed landmark coordinates were independent of the size of shell, which is strongly correlated with the age of an individual (Congdon *et al.*, 2003). We collected four sets of landmarks separately: carapaces and plastrons of females and males. For carapaces, analysis was conducted only for native populations owing to the small eligible sample size (four individuals) from the introduced population (CZ).

To assess the morphological variation in populations, we used canonical variate analysis (CVA). This method calculates canonical variate (CV) scores at different canonical axes that allow discrimination between defined populations. The patterns of morphological variation among populations were then visualized in scatter plots. Pairwise differences in mean shape among populations were tested using the permutational procedure based on Procrustes distances implemented in MORPHOJ (10 000 permutations). Changes in carapace and plastron shape from the overall average shape associated with canonical variates were visualized using wireframe graphs. Furthermore, in MORPHOJ software we performed a Procrustes ANOVA with body side as fixed and individual as a random effect to evaluate asymmetry in the shape of dorsal and ventral shell structures as independent variables (Klingenberg & McIntyre, 1998). For modelling the effects of sex and population (fixed factors) on Procrustes fluctuating asymmetry scores of shell structures, we used the general linear model with a Gaussian error distribution, identity link function and type III sum of squares, with post-hoc Student's paired *t*-tests for significant differences among all pairs of populations compared using the R package 'car' v.3.0-2 (Fox & Weisberg, 2019). Finally, in the R package 'geomorph' v.3.3.1 (Adams *et al.*, 2020) we measured morphological disparity among different populations using Procrustes variance, calculated as the sum of the diagonal elements of the group covariance matrix divided by the number of observations in the group.

RESULTS

FREQUENCY OF SHELL ANOMALIES IN POPULATIONS

Anomalies were found in carapaces only, and we did not find any individuals with multiple abnormalities. The frequency of particular types of anomalies differed significantly among study populations ($\chi^2 = 27.3$, d.f. = 12, $P = 0.007$). Most individuals had shells without accessory scutes or abnormal shell or scute shape, but some populations had significantly higher proportions of individuals that deviated from normal. Differences were found between marginal or isolated populations (SK and CZ) and populations from the core range in

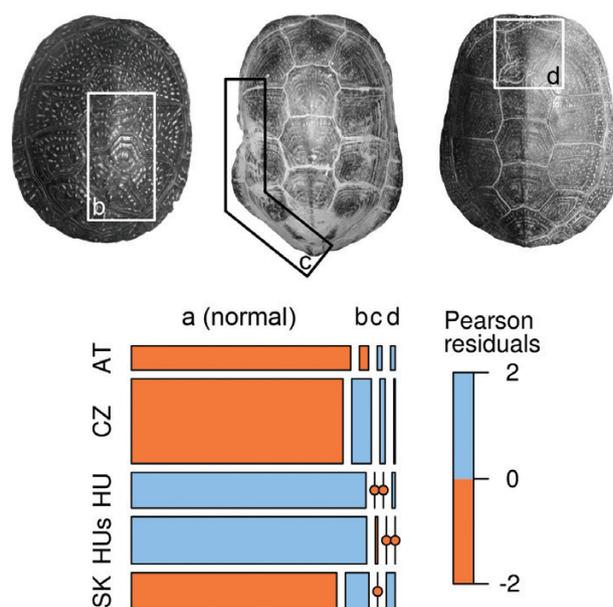


Figure 3. Proportions (mosaic plot) and examples (photographs) of carapace anomalies in different *Emys orbicularis* populations, as defined in Figure 1 (a, normal shell; b, accessory scute; c, abnormal shell shape; d, abnormal scute shape). Each tile is coloured to show the deviation from the expected frequency (residuals) from Pearson's χ^2 test (negative, significantly less or positive, significantly more than expected).

the study area (HU and HUs). Hungarian samples had almost no individuals with anomalies (Fig. 3).

MORPHOLOGICAL SEPARATION OF POPULATIONS

The CVA scatter plots based upon 18 carapace and 20 plastron landmarks showed that individuals associated with different populations mostly overlapped. The first canonical axis (57.7–73.8% of variation explained according to shell structure and sex) separated imperfectly only the native HUs population from the more marginal populations AT, HU and SK (Fig. 4). A weak morphological separation from the other populations could also be detected in introduced CZ individuals (Fig. 4C, D); however, carapace data from this population were not available. Wireframe graphs demonstrated changes in shape mainly along the craniocaudal axis of both shell structures. Regardless of the sex, the most distinct changes in epidermal scutellation were found in the cephalic region. In this view, HUs individuals had shorter scutes compared with the others and, on the contrary, they had elongated scutes in the caudal region. The second canonical axis did not suggest stronger separation (only 15.3–23.1% of variation explained), except for plastrons of AT females or HUs males (Fig. 4C, D).

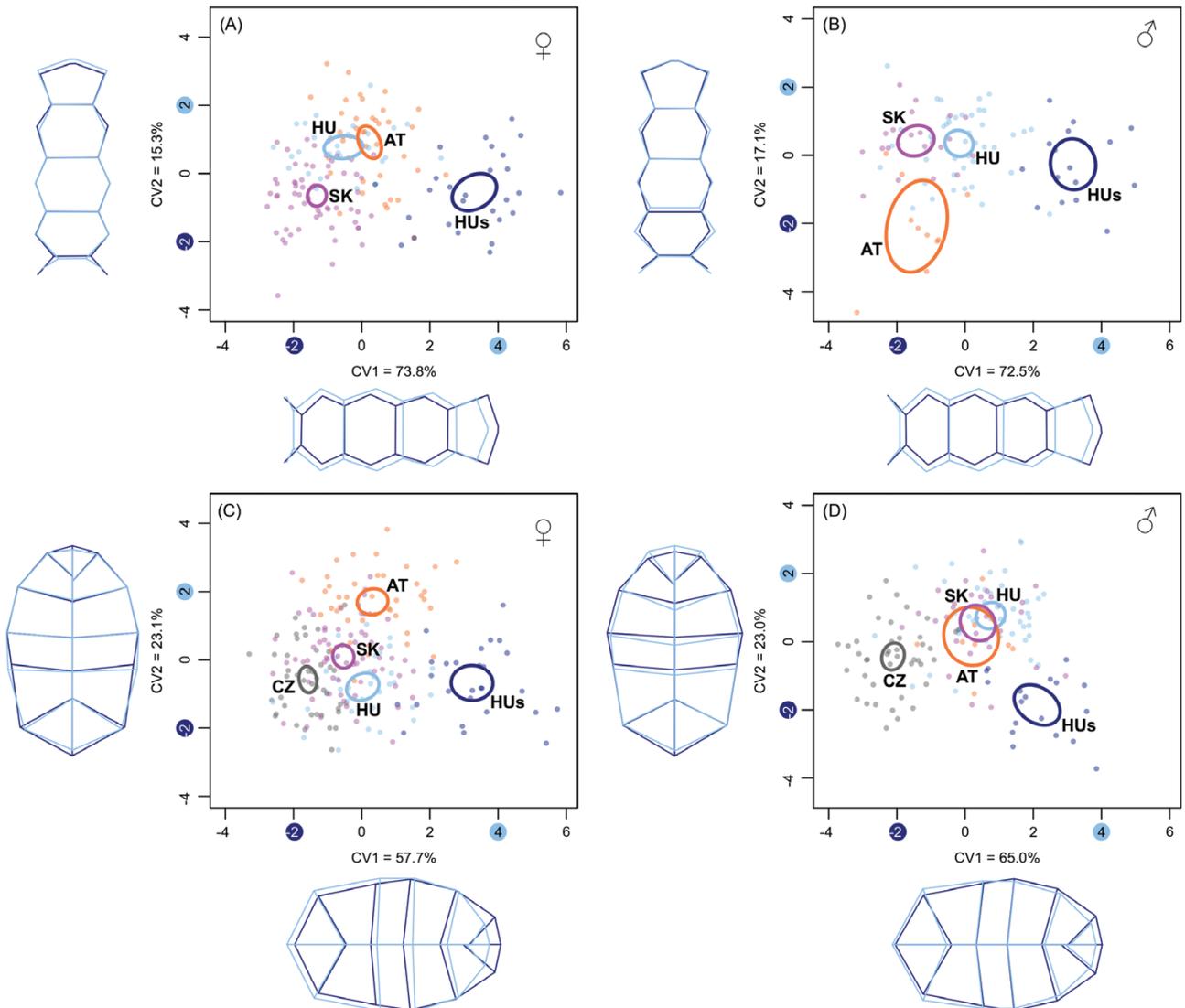


Figure 4. Canonical variate analysis (CVA) plots of morphometric data for carapaces (A, B; 20 geometric landmarks) and plastrons (C, D; 18 geometric landmarks) of five *Emys orbicularis* populations, as defined in Figure 1. Ellipses show 95% confidence of means for each population. The wireframe graphs of shells indicate changes of the shape associated with canonical variate (CV)1 scores of -2 (dark) and +4 (light) and CV2 scores of -2 (dark) and +2 (light).

Permutation tests of the difference in average shape found that only HUs and CZ populations differed significantly from the others ($P < 0.01$) in all pairwise comparisons (Fig. 5). The lowest Procrustes distance was found between HU and SK (Fig. 5C, D), as suggested also by their close position in the CVA scatter plots. The significant effect of body side in Procrustes ANOVA suggested asymmetry within populations for both shell structures (Table 2). In the linear model, variation in the Procrustes scores of plastrons was explained by the population sampled (Table 3). Although the overall model for carapace was not significant, multiple pairwise comparisons revealed similarly significant differences between populations (Fig. 6). Regarding the shape

disparity in different populations, pairwise comparisons of Procrustes variance in plastron data were inconsistent, and thus were not applicable to determine differences among lineages (Table 4).

DISCUSSION

In this study, we examined phenotypic divergence in different *E. orbicularis* populations inhabiting central and marginal parts of their distribution range. Our expectation that more isolated marginal populations (AT and SK) would show higher morphological

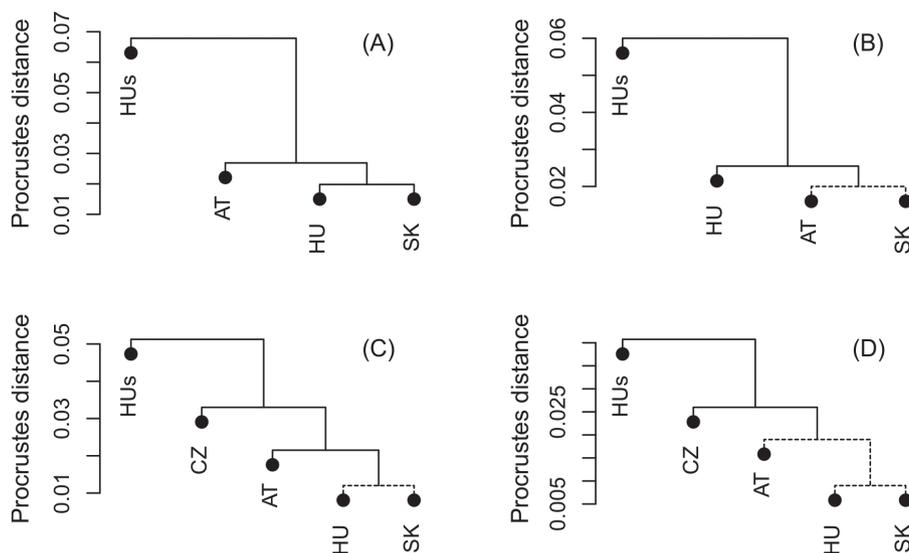


Figure 5. Trees constructed by the unweighted pair group method with arithmetic mean (UPGMA) and based on the matrix of the Procrustes distances between populations, as defined in Figure 1, according to different morphometric data (A, carapaces of females; B, carapaces of males; C, plastrons of females; D, plastrons of males). Significant and non-significant P -values from permutation tests (10 000 permutations) for the null hypothesis of equal means are shown by continuous ($P < 0.01$) and dotted ($P > 0.05$) lines, respectively.

Table 2. Procrustes ANOVAs of shell shape

Effect	Sum of squares	d.f.	F	P -value
Carapace				
Individual	0.686	4410	4.37	< 0.001
Side	0.013	18	19.93	< 0.001
Individual \times side	0.157	4410	–	–
Plastron				
Individual	1.122	5600	7.30	< 0.001
Side	0.007	16	14.88	< 0.001
Individual \times side	0.154	5600	–	–

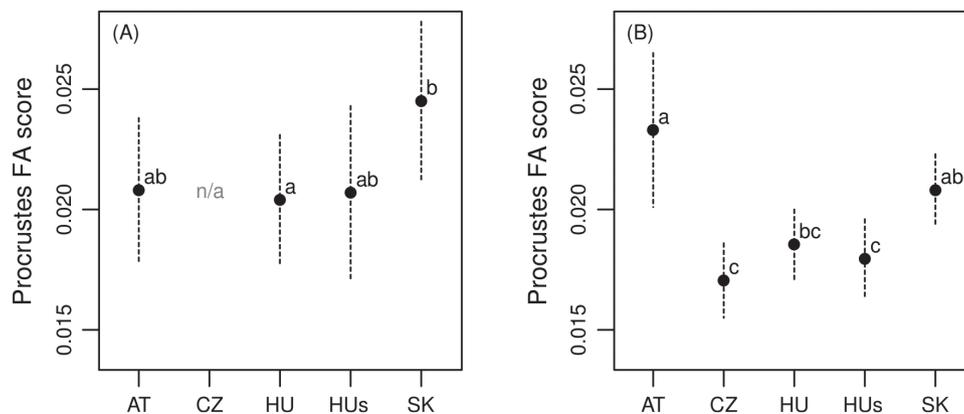
deviations from the core area was supported by higher levels of fluctuating asymmetry in the shell shape. However, observed differences in the shell shape and scute malformations were not a significant predictor of population isolation or of potential environmental stress. Furthermore, the higher proportion of individuals with malformed scutes in the introduced (CZ) and marginal (SK and AT) populations is consistent with other *E. orbicularis* populations where a disrupted population structure was observed. This agrees with the high level of scute anomalies in other populations of this species, where such a pattern was presumably caused by their allochthonous origin and/or a demographic bottleneck and lack of gene flow (Mosimann, 2002; Velo-Antón *et al.*, 2011).

Numerous pholidotic deviations of bottlenecked populations resulting from introduction have been

observed also in other reptiles; for example, in the dice snake (*Natrix tessellata*; Gautschi *et al.*, 2002). Variability in pholidosis in reptiles also depends on the environmental conditions during embryonic development (Yntema, 1960; Telemeco *et al.*, 2013; Moustakas-Verho *et al.*, 2014; Zimm *et al.*, 2017), but a major effect of suboptimal incubation temperatures seems unlikely because the climatic conditions in the study region are similar to other central European parts of the species range (Kottek *et al.*, 2006). Among the populations we had examined, populations in Hungary showed the lowest incidence of shell anomalies. Given that developmental instabilities are also caused by environmental pollution (Bishop *et al.*, 1998; Bell *et al.*, 2006; Cortés-Gómez *et al.*, 2018; Nagle *et al.*, 2018), our results might indicate both favourable genetic conditions and favourable

Table 3. Linear models of Procrustes fluctuating asymmetry scores of dorsal and ventral shell structures explained by two factors and their interaction

Effect	Sum of squares	d.f.	<i>F</i>	<i>P</i> -value
Carapace				
Sex	0.001	1	0.10	0.757
Population	0.001	3	1.63	0.183
Sex × population	0.001	3	0.56	0.642
Residuals	0.038	238	–	–
Plastron				
Sex	0.001	1	0.82	0.366
Population	0.001	4	6.02	< 0.001
Sex × population	0.001	4	0.56	0.694
Residuals	0.020	341	–	–

**Figure 6.** Variation of fluctuating asymmetry (FA; means and 95% confidence intervals) in different populations, as defined in Figure 1, and shell structures of the carapace (A) and plastron (B). Groups sharing a letter are not significantly different ($\alpha = 0.05$) according to post hoc Student's paired *t*-test.

environmental conditions in this part of the species range. Significantly reduced water quality caused > 14% of malformed individuals in a population from the same area (Balázs & Györfy, 2006).

Contrary to Aleksić-Kovačević *et al.* (2014) and Nagle *et al.* (2018), who reported significant shell malformations and macroscopic changes of the skeletal system in the majority of examined turtles, we detected only minor scute and shell shape anomalies in all the studied populations. This might be caused by the different methodology and criteria for determination of shell anomalies or inclusion in the dataset of juveniles, which are known to be more subject to phenotypic changes, such as asymmetric scute growth (Moustakas-Verho *et al.*, 2014; Zimm *et al.*, 2017). We had analysed only adults; thus, we might expect that hatchlings with a normal shell are more likely to reach maturity than individuals with deformities (Mast & Carr, 1989; Telemeco *et al.*, 2013).

Nevertheless, our results provided some relative measures of shell deformities in different populations, and it would appear that minor changes have no significant effect on their viability, but a selective disadvantage of shell anomalies cannot be excluded completely.

Among the other factors that could influence plasticity of the shell shape in turtles is resource availability and/or the quality of different habitat features (Ceballos *et al.*, 2014; Zuffi *et al.*, 2017). Various hydrodynamic conditions of the habitat, mainly differences between lentic and lotic regimens, have also been shown to influence intraspecific variation of carapace size and shape (Lubcke & Wilson, 2007; Rivera, 2008; Rivera *et al.*, 2014). In our study, although the studied populations are distributed over a relatively large geographical region, they inhabit almost identical freshwater habitats; therefore, the effect of flow regimens and habitat quality on variation

Table 4. Comparison of morphological disparity according to plastron between different populations

Population	Procrustes variance	Pairwise differences (<i>P</i> -values)			
		AT	CZ	HU	HUs
Females					
AT	0.0025	–	–	–	–
CZ	0.0040	0.176	–	–	–
HU	0.0018	0.549	0.048	–	–
HUs	0.0028	0.788	0.346	0.423	–
SK	0.0041	0.138	0.966	0.030	0.268
Males					
AT	0.0016	–	–	–	–
CZ	0.0024	0.684	–	–	–
HU	0.0034	0.292	0.362	–	–
HUs	0.0012	0.807	0.392	0.106	–
SK	0.0041	0.168	0.169	0.586	0.060

Sampling sites of *Emys orbicularis* were as follows: AT, eastern Austria; CZ, Czechia; HU, central Hungary; HUs, southeastern Hungary; SK eastern Slovakia.

in shell shape is less likely. Although the introduced CZ population showed significant differentiation in average shell shape, we would not attribute this result to a different phylogenetic origin because it was also found in other species (Chiari *et al.*, 2009; Álvarez-Varas *et al.*, 2019). Surprisingly, the core range population in southern Hungary inhabiting Lake Fehér was the most differentiated, whereas marginal populations in Slovakia and Austria clustered according to plastron data even with the Hungarian population in the Danube catchments area. Together with the lack of morphological disparity in distinct phylogeographical lineages, this pattern suggests that geographical variation is not a function of distance but is the result of limited gene flow (Farkas *et al.*, 1998).

Higher estimates of fluctuating asymmetry in the two marginal populations (AT and SK) compared with the core populations from Hungary seem to be a valuable indicator of their isolation. Thus, our results mirrored population location with respect to the range periphery, which could, in general, support the central–marginal hypothesis. High levels of FA in isolated populations were also revealed in fish and two lizard populations (*Pungitius pungitius*, *Podarcis bocagei* and *Podarcis hispanica*; Băncilă *et al.*, 2010; Trokovic *et al.*, 2012), but unfortunately, such studies are lacking for turtle populations. Increased FA might also indicate higher levels of genetic or environmental stress (Graham *et al.*, 2010), and given that there is no information available about the genetic structure of the studied populations, an effect of inbreeding depression cannot be ruled out, especially given that increased inbreeding and genetic drift were detected in several isolated reptile populations (Clark

et al., 2011; Gallego-García *et al.*, 2018). Moreover, a combination of inbreeding or reduced heterozygosity and habitat fragmentation increases FA (Sarre, 1996; Lens *et al.*, 2000; Eterovick *et al.*, 2016). Even in the absence of genetic data, inhabiting the periphery of the species range makes populations generally more prone to genetic stress owing to inbreeding and genetic drift (Eckert *et al.*, 2008). Depleted genetic variation could be also caused by their isolation from a source of immigrants from nearby populations (Garner *et al.*, 2004). The Danube Delta, the site of origin of the reintroduced Czech population, represents one of the hot spots of *E. orbicularis* distribution in Romania and is situated in the centre of the species distribution range (Rogner, 2009; Birsan *et al.*, 2017). For this reason, the low FA of the introduced Czech population can be explained by the ability of this long-lived species to maintain high genetic diversity (Kuo & Janzen, 2004) and also by their long generation time (Fritz, 2012); therefore, the expression of significant changes might require a greater number of generations from the time when they were reintroduced (Marsack & Swanson, 2009). This would then be consistent with findings that morphological variations in the shell shape between populations reflect historical adaptations more than current gene flow (Clavijo-Baquet *et al.*, 2010).

In conclusion, our morphological analysis provides initial insights into the degree of population isolation of *E. orbicularis* populations in central Europe. Based on these results and on the tendency for population decline, especially of the Slovak population, more detailed information is needed about their genetic status, which might contribute to the initiation of a more effective conservation policy design. In

addition, for a better understanding of the role of the environment in the phenotypic variation in turtles, further studies should focus on habitat quality and the development of pond turtle embryos in different environmental conditions within the species range.

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