Carapace shape variation of genetically divergent populations of *Testudo hermanni boettgeri* (Reptilia: Testudines)

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Abstract: The published *cytb* mtDNA sequences from a previous phylogeographic study of *Testudo hermanni* and *de novo* genetic and phenotypic data of samples from the Pčinja River Valley (Serbia, Central Balkans) and Trebinje (Bosnia and Herzegovina) were used to: (i) genetically characterize samples from Pčinja, a region of great importance from the conservation standpoint due to its taxonomic diversity and biogeographical history, and Trebinje, a type locality of a disputed taxon "*Testudo hercegoviensis*" Werner, 1899; (ii) to study the link between gene genealogy and geographic distribution by implementing spatial genetic analyses, and (iii) to assess whether the distribution of carapace shape variation is in accordance with genetic clusters. The samples from Pčinja and Trebinje possessed divergent haplotypes that corresponded to east (HI) and west (HV) genetic clusters, respectively. Geometric morphometrics was found to be a suitable method to distinguish divergent genetic clusters, arguing for the reassessment of a subspecific ranking within the currently recognized eastern Hermann's subspecies, *T. h. boettgeri*.

Keywords: *cytb* mtDNA; geometric morphometrics; landscape genetics; subspecies; turtle

INTRODUCTION

The rapid loss of biological diversity is indisputably linked to environmental perturbations and extensive habitat degradation caused by human activities [1]. For instance, global climate change, habitat loss and degradation, introduced invasive species, the pet trade, environmental pollution, disease and parasitism, and unsustainable exploitation are considered to be the main anthropogenic causes for the worldwide decline in reptiles [2]. However, many species that suffer from negative anthropogenic impact have neither been recognized nor consequently monitored due to the notable lack of sufficient data about their distribution and ecology, as well as their taxonomical and conservation status [2]. Within reptiles, the substantial declines in freshwater turtles and tortoises have been recognized as a global turtle survival crisis since 56.3% of 251 species with IUCN Red List status are considered as Threatened [3]. One of the key challenges in turtle conservation includes the disputed taxonomical status of taxa, which consequently underestimates the actual number of threatened taxa of the focal region [1-3].

The Hermann's tortoise, *Testudo hermanni* (Gmelin, 1789), is one of three tortoise species that inhabit the European part of the Mediterranean basin [4,5], a region known for its significant levels of biodiversity and rich taxonomic uniqueness [6]. Currently, the Hermann's tortoise is globally listed as Near Threatened according to the IUCN's Red List [7], although an update of its IUCN conservation status has been suggested [4,8]. In addition, the species is protected under the CITES regulative (Appendix II), Habitat Directive (Annexes II and IV), and Bern Convention (Annex II), as well [8,9]. Major threats to the species are habitat degradation, hazardous wildfires, harvesting for the pet trade and road mortality, as well as agriculture and urbanization in the coastal part of the Mediterranean region [4,10,11]. Even large and dense populations seem to be prone to an uncertain viability over the years [8].

The nomenclatural history of this species is complex [12], however two subspecies are currently recognized: *T. h. hermanni* (Gmelin, 1789) and *T. h. boettgeri* (Mojsisovics, 1889). Unlike *T. h. hermanni*, which occurs in eastern Spain, southern France and Italy (including the Balearic Islands, Corsica, Sardinia and Sicily), *T. h. boettgeri* inhabits the southern and central parts of the Balkan Peninsula [4]. The two-subspecies proposition was established by the work of Wermuth [13], but this division was later challenged. Due to the absence of inguinal scutes, populations of *T. h. boettgeri* along the Adriatic coast from northern Croatia to southern Montenegro (up to the River Bojana) were suggested as a distinct species, *T. hercegovinensis* [14,15]. The work of Perälä [14,15] was based on the previous study by Werner [16] who showed that individuals from the vicinity of Trebinje (Bosnia and Herzegovina) represent a distinct morphological variant of the nominal species and recognized them as *Testudo graeca* var. *hercegovinensis* [16]. Therefore, according to Perälä [14,15], nominal *T. hermanni* should be split into three species (*T. hermanni*, *T. hercegovinensis* and *T. boettgeri*), with some authors supporting this division [17-19]. Fritz et al. [20] used a model-based phylogenetic inference and parsimony network analysis to study the phylogeographic structure of the species based on molecular variation of *cytb* and the adjacent *tRNA-Thr* gene of mitochondrial DNA (mtDNA). Although the latter method has been shown to be more sensitive to detection of genetic structuring within the eastern subspecies *T. h. boettgeri*, the phylogenetic approach was used in defining the subspecies status as the presence/absence of monophyletic groups on a phylogenetic tree [20]. Therefore, the three-species model [14,15] has been discarded and the use of the traditional two-subspecies model [13] was suggested [20]. The Balkan populations were grouped in three allopatric haplogroups (B1-B7: central, eastern and southern parts of the Balkans; B8-B9: western slope of the Taygetos Mts., southern Peloponnese; and B10-B15: Adriatic coast from northern Croatia to Epirus in Greece), which were suggested as distinct Evolutionarily Significant Units (ESU) but without recognized taxonomical rank [20]. The same study claimed that the lack of inguinal scutes was not the unique feature of "*T. hercegovinesis*", which additionally supported the two subspecies division as proposed by Wermuth [13,20].

The variation within *T. hermanni* has been studied by assessing geographically distinct samples using molecular markers and a traditional morphological approach (qualitative traits and linear morphometrics), given that the allopatric distribution, a certain degree of genetic divergence and morphological differentiation between characteristic sets of conspecific populations represent the hallmarks of a polytypic species [21-24]. However, in line with the proposition for further research on this taxon [4], a cutting-edge method such as geometric morphometrics, proven to be powerful in the detection of subtle phenotypic variation [25], has not been applied in studies of morphological differentiation of genetically recognized groups within this species. The study by Fritz et al. [20] is the most comprehensive phylogeographic study of this species and has consequently been widely used as a basis for the current taxonomical ranking of Hermann's tortoise populations. However, due to the taxonomic ambiguity and putative cryptic diversity, both harboring consequences for conservation management strategy, the Balkan's *T. hermanni* populations should be further investigated in greater detail.

Given the conservation and taxonomical importance of the detection of cryptic diversity within *T. hermanni boettgeri*, we addressed three specific aims. First, we studied the molecular variation of *cytb* mtDNA of *T. h. boettgeri* sampled from two populations (Pčinja River Valley, Serbia and Trebinje, Bosnia and Herzegovina) that have not been included in the rangewide phylogeography study by Fritz et al. [20]. The two chosen localities are of importance since Trebinje is a type locality of the disputed taxon *T. greaca* var. *hercegoviensis* (Werner, 1899), while Pčinja River Valley is a region of great importance from the conservation perspective due to its taxonomic diversity and biogeographical history [26-29]. Second, we analyzed a joint dataset (sequences are from Fritz et al. [20] and our *de novo cytb* mtDNA sequences) using a spatially informed Bayesian clustering specifically designed for individual- and population-level analyses [30,31] in order to complement the current knowledge of the species' phylogeography. Finally, by implementing comprehensive morphometric analyses, we investigated whether the carapace shape sufficiently differs between populations from Pčinja River Valley and Trebinje. Our data provide new insights into the current knowledge of the intraspecific diversity within *T. hermanni* across its range, with conservation implications.

MATERIALS AND METHODS

Sampling and field procedures

In this study, we analyzed 99 free-living adult specimens with fully developed and undamaged shells, collected from two localities, Trebinje (Bosnia and Herzegovina) and Pčinja River Valley (Serbia) (Supplementary Table S1). In order to infer the membership of Trebinje and Pčinja specimens to previously described haplogroups, we also included 87 sequences reported by [20] (Supplementary Table S2).

Adult individuals are those whose straight carapace is longer than 100 mm [32], and sexes were identified based on sex-specific macromorphological characteristics, such as tail length, concavity of the plastron, anal notch width and the curvature of posterior peripheral plates [5]. The collection and temporary handling of tortoises were regulated by permissions issued by the Ministry of Environmental Protection of the Republic of Serbia (119-01-5/17/2014-09), while such regulations were not required for Bosnia and Herzegovina due to a lack of conservation status and protection of the species in Republika Srpska. The field protocol included sex determination of specimens, labelling the individuals, image acquisition, blood collection and georeferencing. In the field, each individual was photographed twice from the dorsal (carapace) perspective in order to estimate imaging error. Images were obtained using a hand-held NIKON COOLPIX S8000 camera with automatic settings (14.2 MP resolutions), positioned perpendicular to each individual. Photographs were obtained by the same person (MĐ). The imaging system was accompanied by a millimeter scale, later used for size calibration, and a bubble level that ensured the absence of inclination of the system relative to the field ground. Blood samples were obtained by puncturing the jugular veins of the tortoises, collecting a small amount of blood (max 300 μL) and storing it on FTA® cards (Whatman, Part of GE Healthcare, USA). For each individual, GPS coordinates of the exact capturing location were obtained.

DNA extraction and amplification

From each individual blood sample stored on FTA® cards, we punched three to five 1.2-mm disks using the Harris micro-punch and a cutting mat. Cross-contamination between punches was prevented by rinsing the cutting mat and the micro-punch with ethanol between sampling. Likewise, the micro-punch was dried by punching a disk from an unused FTA® and common filter paper. Genomic DNA was extracted with a NucleoSpin® Tissue kit (Macherey-Nagel, Germany) using a protocol for dried blood spots. A fragment of 750 bp, containing both ends of the *cytb* mtDNA gene, was amplified using the following primers: L 14722 (forward direction; 5'-CGAAGCTTGATATGAAAAAC-CATCGTTG-3') [33] and H 15231 (reverse direction; 5'-GCAAATAGGAAGTATCATTCTGG-3') [34]. PCR amplification was performed using the Illustra™ PuRe-Taq™ Ready-To-Go™ PCR kit (GE Healthcare, USA) under the following conditions: initialization at 94°C for 5 min, then 40 cycles of denaturation at 94°C for 45 s, annealing at 53 °C for 45 s, elongation at 72°C for 90 s, and a final elongation step at 72°C for 10 min [34].

Using horizontal electrophoresis (1.5% agarose gel with ethidium bromide), we checked the PCR products and afterwards purified them with ExoSAP-IT® (USB Corporation, USA) in a two-step reaction (37°C for 30 s and 80°C for 15 s), following the manufacturer's protocol. Finally, purified PCR products were sequenced using the ABI prism Big Dye Terminator Cycle sequencing kit (version 3.1) on an ABI 3730xl DNA Analyzer (Applied Biosystems, USA). Each individual PCR product was sequenced in both the forward and reverse direction. DNA sequences were manually checked by the same person (VM) for errors in Chromas Lite (v.2.1.1.) (Technelysium Pty Ltd, http://www.technelysium.com.au).

DNA sequence analyses

All sequences were aligned by ClustalW algorithm in MEGA X [35]. Relative to the one reported in [20], the sequence frame we amplified was about 150 bp longer and approximately 400 bp shorter in the 5' and 3' directions, respectively. The shared sequence frame was about 600 bp long, and after trimming the alignment by excluding invariable sites at the both ends of the frame, the final length of all sequences analyzed was 518 bp. In order to infer affiliation of our samples relative to the four haplogroups provided in Fritz et al. [20], a haplotype network [36] in the *pegas* package v.0.11 [37,38] was constructed for the R statistical language. The landscape genetics of *T. hermanni* was

assessed with the Geneland package v.4.0.8 [30,39] for the R statistical language, which is the Bayesian clustering method that can explicitly consider the spatial structure of the data. The main aim of this method was to determine the number of populations that maximize the Hardy-Weinberg equilibrium and the linkage equilibrium within them. Because of the lack of spatial information, the sequence AJ888364 [20] was excluded from the analysis and the whole input dataset consisted of 96 georeferenced samples. Variable substitutions found in our sequence alignment were extracted in MEGA X and nucleotides were coded as T=1, C=2, G=3, A=4. The latitude and longitude coordinates of each individual (spherical coordinates) were transferred to planar coordinates (UTM; Geneland requirements for the spatial structure inference). Allele frequencies in Geneland can be treated according to both uncorrelated and correlated models [30,39]. For the MCMC procedure, we set 10 independent runs, each with 100000 iterations, and the chains were thinned every 100 iteration; burning was set to 200; the maximum rate of the Poisson process was 96 (equal to the number of individuals); the maximum number of populations was set to 10; the maximum number of nuclei in the Poisson-Voronoi tessellation was 288 (three times the number of individuals, as suggested in the Geneland manual). We used the spatial model without assuming uncertainty of spatial coordinates and genetic data were treated as haploid data (standard procedure for mtDNA) [40]. A convergence of MCMC was tested by visual inspection of a trace plot of 10 independent runs for both uncorrelated and correlated models of allele frequencies. Likewise, we compared the consistency of the estimated number of populations (K) and individuals' affiliation to those clusters across independent runs and two models.

Geometric morphometric analyses

On each image, 2D Cartesian coordinates of 28 fixed landmarks were digitized by the same person (MDj) using tpsDIG (v. 2.17) [41] (Supplementary Fig. S1). Landmarks were digitized on the intersections of dermal scutes (keratinized epidermal structures or dermal plates) and the distribution of landmarks covers vertebral (neural) and costal scutes of the carapace. Twenty-six landmarks were paired (1-26), while two (27, 28) were unpaired and they define the longitudi-

nal axis that divides paired landmarks. The carapace shape was analyzed using landmark-based geometric morphometrics [42]. Shape variables (Procrustes tangent coordinates) were obtained by generalized Procrustes superimposition [43], which mathematically removes the effects of non-shape variation (position, orientation and isometric size). As all individuals were photographed twice, prior to analyses of shape variation, an assessment of the effect of imaging error was checked with Procrustes analysis of variance (ANOVA) for object symmetry [44]. The symmetric component of shape variation for each individual was extracted and used in subsequent analyses. The use of the symmetric component of shape variation is particularly important for structures with object symmetry, as it improves the accuracy of shape estimates by "averaging" between body sides using mirror-reflection [25,44,45]. Centroid size (CS) was used as a geometric measure of size. The superimposition, Procrustes ANOVA for bilateral symmetry and extraction of the symmetric component of shape variation and CS for each specimen were performed in the geomorph package (v. 3.1.2) [46,47] in R (v. 3.6).

The relative contribution of the logarithm of centroid size (hereinafter referred by the term "Size" for Log (CS)), Sex and Haplotype to the total shape variation was assessed using the linear models performed in geomorph [46] and RRPP R packages applying randomized residuals in a permutation procedure (RRPP) when applicable using 1000 iterations [47]. This procedure is particularly appropriate for this study since it can evaluate the relative contributions of each term in the linear model relative to the total amount of shape variation, which is expressed as a coefficient of determination (R^2); this method is not sensitive to cases where the number of variables is greater than the number of individuals; it provides Z scores that are the measures of an effect size of particular term (e.g. the standard deviation of an effect sum-of-squares relative to the random distribution of sum-of-squares generated by permutations [48]). Using Z scores as an effect size allows for the comparison of results across studies. It is important to note that the sum-of-squares of terms in the models was assessed using Procrustes distances among specimens, rather than the explained covariance matrices among variables. We performed principal component analysis (PCA) to observe distribution of individuals in the morphospace defined
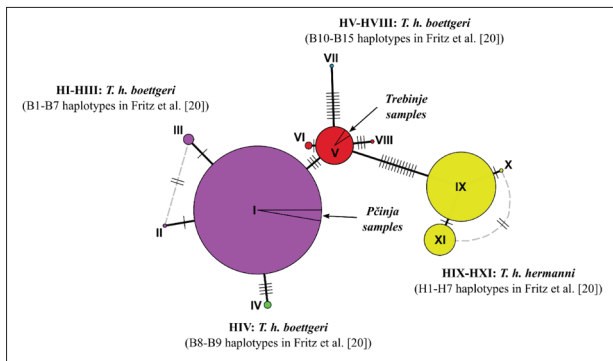
Fig. 1. The haplotype network, based on *cytb* mtDNA sequence variability of *Testudo hermanni boettgeri*, showing the positions of the Pčinja and Trebinje samples relative to the four haplogroups of *Testudo hermanni* defined in Fritz et al. [20], depicted with different colors. Dashed lines indicate alternative reticulation between individual haplotypes and vertical lines represent the number of substitutions between haplotypes.

by the first two PC vectors. Shape changes between haplotypes within each sex were visualized by warped outline drawings [49]. Accuracy of discrimination between haplotypes based on carapace shape was established by discriminant function analysis (DFA) using leave-one-out cross-validation. Warped outline drawings and the percentage of correct classification from DFA were obtained in MorphoJ (v. 1.06d). PCA was conducted in R, while warped outline drawings and the percentage of correct classification from DFA were obtained in MorphoJ (v. 1.06d) [50].

RESULTS

Molecular characterization and landscape genetics

Eleven *cytb* mtDNA haplotypes were found in *T. hermanni* species, three (HIX-HXI) and eight (HI-HVIII) of which were registered in *T. h. hermanni* and *T. h. boettgeri* subspecies, respectively (Supplementary Tables S1 and S2). Common haplotypes found in *T. h. hermanni* were HIX and HXI. Common haplotypes in *T. h. boettgeri* characterize populations along the eastern Adriatic Sea coast and northern Ionian Sea, including Croatia and Epirus in Greece (HV), as well as the continental part of the Balkans with the majority of Greek populations (HI). In *T. h. hermanni*, a unique haplotype (HX) was found in one out of seven individuals from Apulia in Italy. In *T. h. boettgeri*, six unique haplotypes were identified, all registered in Greece: Macedonia
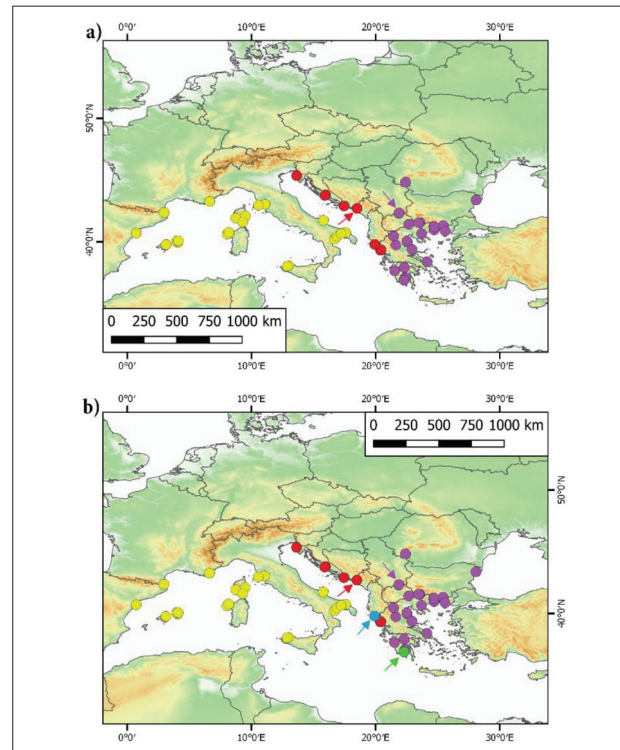
Fig. 2. The posterior probability of population membership of *Testudo hermanni* samples inferred by Geneland depicted on the map of Europe found through (**a**) an uncorrelated model and (**b**) a correlated model. Purple and red arrows indicate the locations of samples from the Pčinja River Valley and Trebinje, respectively. Blue and green arrows indicate individuals from Corfu and Saidhona in Greece, respectively. Colors match with the haplogroup depiction in Fig. 1.

(HII), Evvia (HIII), Peloponnese (HIV), Parga (HVI, HVIII), and Corfu (HVII). Haplotypes represented with a single individual in the present study were HII, HVII and HVIII within *T. h. boettgeri*, as well as HX within *T. h. hermanni* (Fig. 1). Eight tortoises analyzed from the Pčinja River Valley (Serbia) belonged to the most prevalent haplotype found in the continental part of the Balkans (HI), while two individuals from Trebinje (Bosnia and Herzegovina) represented the HV haplotype, specific to the eastern Adriatic Sea coast (Fig. 1, and Supplementary Table S2).

Our reconstructed haplotype network showed the presence of three haplogroups, rather than the four as in [20] (Fig. 1). The Bayesian inference on the geo-referenced *cytb* mtDNA dataset in Geneland showed the presence of three to five clusters across the range of Hermann's tortoise (Supplementary Table S2 and Fig. 2). Namely, the model with uncorrelated allele

frequencies systematically recovered three clusters in all 10 independent runs (Fig. 2b): (i) cluster A, which consisted of populations of *T. h. hermanni* found in Spain, France, and Italy; (ii) populations across Croatia and Bosnia and Herzegovina and northern Greece (Corfu and Epirus) in the proximity of the Adriatic and Ionian seas, respectively, were attributed to cluster B; (iii) populations from Romania, Serbia, Bulgaria, the Republic of North Macedonia and Greece were attributed to cluster C. In the model where allele frequencies were assumed to be correlated, four and five clusters were supported, each with five independent runs (Fig. 2b). In general, estimated clusters in uncorrelated and correlated models do match, but with slight differences. In five runs where four clusters were found, either the individual AM230530 from Corfu (four out of five runs) or two individuals from Saidhona in Peloponnese, Greece (AM230535 and AM230536; one out of five runs) were attributed to distinct clusters. Likewise, the same individuals were distributed to clusters D and E (Supplementary Table S2) in the remaining five runs under the correlated model of allele frequencies.

Geometric morphometric assessment of populations from genetically divergent clusters

After characterizing the molecular variation of individuals from Pčinja (haplotype HI) and Trebinje (haplotype HV), we studied the pattern of carapace shape variation, employing geometric morphometric techniques. First, we checked the effect of measurement error (in this case an imaging error) using Procrustes ANOVA for the object symmetry. It appeared that the imaging error was negligible (3.3%) and with a lower magnitude relative to all hierarchically upstream effects in the model (4.7 and 11.2 times the effects of fluctuating asymmetry and individual variation were higher than the error term (Supplementary Table S3). Hence, the individual variation with 88.1% contributed the most to the total carapace shape variation, as well as having an 11.2-fold larger effect than that of fluctuating asymmetry (Supplementary Table S3). In all subsequent morphometric analyses, replicates were averaged within each individual, which is a standard procedure. The overall pattern of carapace shape variation was studied with a model that tests the effects of tortoise size, sexual shape dimorphism and haplotype membership, as well as their interactions. We found that size, haplotype and sex were significant terms in the fit1 linear model (Table 1). The profound effect on carapace shape was due to sexual dimorphism (29%), followed by differences between haplotypes (5.2%), while the common allometry was subtle (8.6%). We did not observe haplotype-specific or haplotype-sex-specific allometric trajectories (see the "Size:Haplotype" and "Size:Haplotype:Sex" interactione, respectively, in Table 1), but allometric trajectories were different between sexes, though very subtle (see the "Size:Sex" interaction in Table 1). The carapace sexual shape dimorphism was consistent in two haplotypes (populations) (see the "Haplotype:Sex" interaction in Table 1). Given the significant allometric effect as well as the presence of sexual shape dimorphism in the data (Table 1), we assessed whether haplotypes differ in shape after removing the shape variation explained by Size and Sex factors. After checking for both common allometry and common sexual shape dimorphism, haplotypes differed in their carapace shape (Procrustes distance=0.0259, Z=2.8, *P*=0.006). Additionally, we performed the same analyses within each sex without considering allometric effect (Table 2) and considering common allometry (Table 3). Regardless of the models used, haplotypes consistently differed in their carapace shape (Tables 2 and 3) and haplotype-specific allometries were not observed in either sex (Table 3). Finally, it appeared that haplotype affiliation explained more carapace shape variance in females than in males (see R^2 values in Tables 2, 3), indicating that females were more divergent than males.

The distribution of the total carapace shape relative to sex and haplotype was depicted in the morphospace defined by the first two PC axes that accounted for around 60% of the total shape variation (Fig. 3). The sexual shape dimorphism was noticeably the main source of shape variation in the data as the PC1 vector (48.4%) separated the sexes. The shape variation explained by the haplotype was spanned along the PC2 vector that accounted for 11.2% of the total shape variation. The subtle, but significant difference between populations (haplotypes) that were magnified three times for the visualization purpose, is shown in Fig. 3. Females of the two populations differed in the posterior part of the carapace, where females from Trebinje (HV) had a wider posterior

Table 1. Carapace shape variation of *Testudo hermanni boettgeri* structured across two factors (Haplotype and Sex affiliation) and their interaction where logarithm of centroid size (Log (CS)) was used as covariate. Statistical significance of the linear model was evaluated by residual randomization in permutation procedures (RRPP) with 1000 iterations.

Term	Df	SS	MS	R²	F	Z	P
Log (CS)	1	0.0173	0.0173	0.086	14.47	3.69	0.001
Haplotype	1	0.0106	0.0106	0.052	8.83	3.12	0.002
Sex	1	0.0585	0.0585	0.290	48.93	5.81	0.001
Log (CS) : Haplotype	1	0.0008	0.0008	0.004	0.70	-0.46	0.675
Log (CS) : Sex	1	0.0036	0.0036	0.018	2.99	2.52	0.01
Haplotype : Sex	1	0.0010	0.0010	0.005	0.79	-0.11	0.567
Log (CS) : Haplotype : Sex	1	0.0009	0.0009	0.005	0.78	-0.10	0.538
Residuals	91	0.1088	0.0012	0.540			
Total	98	0.2015					

Df – degrees of freedom; SS – sum of squares; MS – mean squares;
R² – coefficient of determination; F – F value; Z – Z scores;
P – significance after 1,000 permutations

Table 2. Total carapace shape difference between haplotypes of *Testudo hermanni boettgeri* within each sex (without considering allometric effect) inferred by permutation procedure using 1000 iterations.

	Term	Df	SS	MS	R²	F	Z	P
Females	Haplotype	1	0.0182	0.0182	0.25	15.58	4.96	0.001
	Residuals	47	0.0550	0.0012	0.75			
	Total	48	0.0732					
Males	Haplotype	1	0.0098	0.0098	0.14	7.67	3.88	0.001
	Residuals	48	0.0616	0.0013	0.86			
	Total	49	0.0714					

Df – degrees of freedom; SS – sum of squares; MS – mean squares;
R² – coefficient of determination; F – F value; Z – Z scores;
P – significance after 1,000 permutations

Table 3. Carapace shape difference between haplotypes of *Testudo hermanni boettgeri* within each sex (considering common allometry). Statistical significance after 1000 permutations.

	Term	Df	SS	MS	R²	F	Z	P
Females	log (Size)	1	0.0081	0.0081	0.111	7.22	3.34	0.001
	Haplotype	1	0.0141	0.0141	0.193	12.53	4.60	0.001
	log (Size) : Haplotype	1	0.0003	0.0003	0.004	0.27	-2.28	0.984
	Residuals	45	0.0507	0.0011	0.692			
	Total	48	0.0732					
Males	log (Size)	1	0.0052	0.0052	0.073	4.10	2.75	0.006
	Haplotype	1	0.0073	0.0073	0.102	5.78	3.61	0.001
	log (Size) : Haplotype	1	0.0008	0.0008	0.011	0.61	-0.60	0.741
	Residuals	46	0.0581	0.0013	0.814			
	Total	49	0.0714					

Df – degrees of freedom; SS – sum of squares; MS – mean squares;
R² – coefficient of determination; F – F value; Z – Z scores;
P – significance after 1,000 permutations
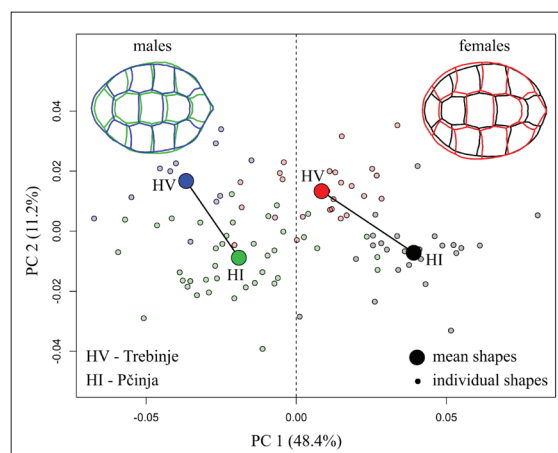
Fig. 3. The carapace shape variation of Pčinja and Trebinje samples depicted with a morphospace defined by the first two PC axes. Vertical dashed line separates the sexes in the morphospace and haplotypes within each sex of *Testudo hermanni boettgeri* are connected by a solid line. The shape differences between haplotypes within each sex are represented with warped outline drawings.

carapace relative to those from Pčinja (HI). In males, the differences between populations were mostly localized on the anteriodistal part of the carapace. In both sexes, it appeared that the first (landmarks 1, 2, 13, 14, 15, 26, 27) and fifth (landmarks 6, 7, 19, 20, 28) vertebral scutes were the most variable features in the carapaces of the two populations. Finally, the percentage of correct classification after leave-one-out cross-validation was higher in females (92%) than in males (81%).

DISCUSSION

Our study confirmed the genetic and morphological distinctiveness of the two populations of *T. hermanii boettgeri* on the Balkan Peninsula. Available 87 *cytb* mtDNA sequences from GenBank [20] that covered the whole range of *T. hermanni,* and the eight and two sequences obtained in this study from the Pčinja River Valley and Trebinje, respectively, were used to test the link between gene genealogy and geographic distribution, as well as to define classifiers for our morphometric analyses. Herewith, the network analysis based on 518-bp-long *cytb* mtDNA sequences identified 11 haplotypes grouped in three main haplogroups. Namely,

HI-HIV and HV-HVIII haplogroups were found within the eastern subspecies *T. h. boettgeri*, while the haplogroup HIX-HXI was found within the western subspecies, *T. h. hermanni*. Using the same sequences but with the length of 1150 bp, Fritz et al. [20] reported 22 haplotypes distributed in four haplogroups. For instance, two individuals found in Saidhona (western slopes of Taygetos Mountain, Southern Peloponnese, Greece) were outlined as the haplogroup B8-B9 [20], while in our study they appeared as a tributary haplotype (HIV) to the most frequent haplotype HI found in *T. h. boettgeri* without forming alternative reticulation with both the HV-HVIII and HIX-HXI haplogroups. Four (36%) and 15 (68%) haplotypes have been defined based on single specimens in this study and that of Fritz et al. [20], respectively. As we trimmed the original sequence alignment [20] for about 50% of its length, the number of haplotypes represented by a single specimen decreased by around 50%, as well. Such an observation is favorable as it supports the argument that (i) the trimming likely did not significantly influence our analyses, e.g. the ability to recover the major patterns in the data reported by Fritz et al. [20], and/or (ii) the molecular characterization of our samples would be simply influenced by shortening the sequence frame. All the samples from the Pčinja River Valley possessed HI haplotype (B1-B7 haplogroup according to Fritz et al. [20]) that widely occurred in *T. h. boettgeri* from Bulgaria, Romania, Republic of North Macedonia and the majority of Greece (Macedonia, Thessaly, Thrace, Thessaly and Peloponnese, except its southern part). Specimens from Trebinje possessed the HV haplotype (B10-B15 haplogroup according to Fritz et al. [20]) which was a common haplotype in populations from the eastern Adriatic coast. We observed a lack of *cytb* mtDNA variation in sample locations from the Pčinja River Valley and Trebinje, which is in agreement with the majority of populations of *T. hermanni* analyzed by Fritz et al. [20]. Hence, our results showed that populations from Pčinja and Trebinje belong to the divergent genetical clusters within the nominal eastern subspecies *T. h. boettgeri*.

As regards to the second aim of this study, landscape genetics was performed to test whether gene genealogy is linked to the geographic distribution of the taxon. By using the uncorrelated model of allele frequencies, three distinct genetic clusters were found that were fully concordant with our haplotype network reconstruction. For instance, clusters A, B and C found in Geneland matched haplogroups HIX-HXI, HV-HVIII and HI-HIV, respectively. Analysis based on the correlated model uncovered two additional genetic clusters within the clusters B and C due to the presence of the unique haplotypes in Corfu (HVII) and Saidhona, Peloponnese (HIV), respectively. It has been shown that the correlated model of allele frequencies is more suitable for recent and subtle genetic subdivisions between populations [39]. However, the correlated model assumes that proximal populations share similar allele frequencies or rare haplotypes among populations, which was not the case in the dataset we analyzed (see previous paragraph). Furthermore, the correlated model is more sensitive to departure from model assumptions such as presence of isolation-by-distance (IBD), which seemed unlikely as the analyzed populations were allopatrically distributed, showing clear phylogeographic structure. In light of these arguments, we conclude that the uncorrelated model of allele frequencies is more suitable for this dataset than the correlated one.

The reported spatial distribution of molecular variation of *T. hermanni* is probably influenced by the biogeographical history of southern Europe. Indeed, the Alps and the Dinaric mountain systems divided populations of the two studied subspecies, as well as western (HV-HVIII) and eastern (HI-HIV) haplogroups within *T. h. boettgeri*. It is worth mentioning that populations from Croatia, Bosnia and Herzegovina and Montenegro were considered as both separate species and subspecies [12]. Furthermore, it has already been suggested that geological history and dramatic climatic changes during the Pleistocene influenced the fragmentation of the ancestral area of *T. hermanni* [20]. As seen in the case of *T. hermanni*, geological barriers accompanied by dramatic climatic perturbations during the Pliocene and Pleistocene are considered to be important factors for biological diversification of the European biota [51,52].

Regarding the utility of geometric morphometrics in distinguishing genetically diverged populations, Pčinja (haplotype HI) and Trebinje (haplotype HV), we showed that they statistically differ in carapace shape although the magnitude of the differences was subtle (overall 5.2%). The upper part of the carapace is a modified rib cage since the vertebral (except anterior

and posterior parts of the first and fifth scutes, respectively) and costal (except their distal parts) plates are fused with the axial skeleton (vertebrae and thoracic ribs) [53]. This is considered to be developmentally conserved across vertebrates [54]. Therefore, subtle divergence in carapace shape between genetically diverged populations might be explained by developmental conservatism of the vertebral and costal bones in turtles that are preserved more than 245 MYA [53]. In addition, we observed that in both sexes divergent genetic units differed the most in the first and fifth vertebral scutes, as well as in the margins of the costal plates. As already mentioned, these carapace regions include some parts that were not directly fused with the axial skeleton, and as such could be more prone to shape modifications. However, much more extensive morphological examinations should be performed to confirm this assumption about constraints that might produce the observed pattern of carapace shape variation. Additionally, future studies could illuminate a possible link between environmental factors and carapace shape variation and evaluate if differences between populations of the same haplotype would be smaller than those between genetically divergent populations. Studying variation in relative body proportions in *T. hermanni boettgeri*, Djordjevic et al. [55] showed that the rear portion of the tortoises' shell and free body parts (e.g. legs, head and tail) were not variable across populations in both sexes. Although partially in agreement with the pattern we observed, their study is not directly analogous with ours due to the following. They compared populations without known genetic background and, based on the provided sampling design, it is likely that they analyzed interpopulation differences within a single genetic cluster (presumably our HI genetic cluster). Finally, morphometric analyses of two studies were different, e.g. univariate vs. multivariate. As such, we emphasize two important aspects of geometric morphometrics: (i) a high potential to detect subtle interpopulation differences due to its conceptual and rigorous statistical properties [25,42], and (ii) the ability to visualize shape changes in an anatomical context that promotes better and more straightforward scientific communication [49]. Furthermore, previous studies disagree on the utility of qualitative characters in the identification of allopatric genetic clusters within *T. h. boettgeri*. Namely, it was proposed that the lack of inguinal

scutes might discriminate populations from historical Dalmatia (our HV haplotype) from other allopatric populations of *T. hermanni boettgeri* [14]. However, it appeared that this qualitative character is not informative in systematically distinguishing respective populations [20]. Therefore, our study demonstrates for the first time a usefulness of quantitative data such as carapace shape in the discrimination of genetically diverged populations in any *Testudo* species.

In summary, Fritz et al. [20] performed the first and the most comprehensive phylogeographic study on this species, and as such provided the core base for the currently accepted subspecific taxonomical ranking in this species [4]. After analyzing the dataset of Fritz et al. [20], along with additional sequences reported in this study using a spatially informed Bayesian inference in Geneland, our results provide additional insights into genetic and morphological variability of Hermann's tortoise. The spatial genetic analysis we performed is more appropriate for individual- or population-based research, which might be more informative in the detection of subtle genetic variation below species level [56]. Finally, our results advocate the need for the taxonomic evaluation of *T. hermanni* complex taxa by integrating multilocus phylogenetic and geometry-oriented morphometrics in order to fully understand the subspecific diversity of the species [57].

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Supplementary Material

The Supplementary Material is available at: http://serbiosoc.org.rs/NewUploads/Uploads/Djurakic%20and%20Milankov_4345_Supplementary%20Information.pdf