

Integrating morphological and genetic data at different climate and geographic scales in an endangered freshwater mussel Unio durieui (Bivalvia: Unionidae) endemic to northern Tunisia

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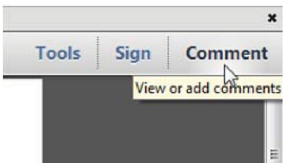
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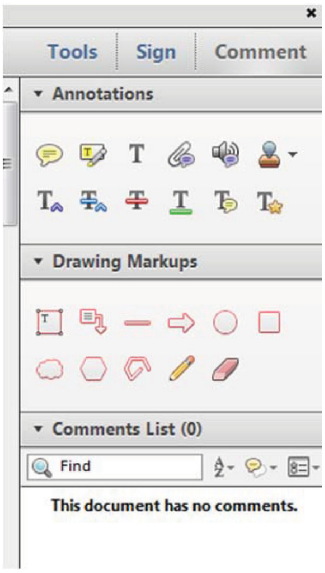
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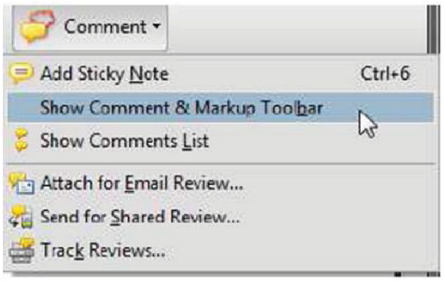


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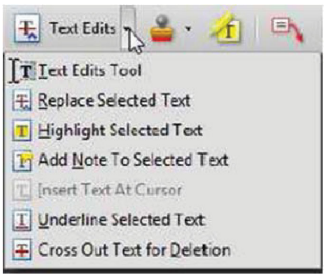
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Integrating morphological and genetic data at different climate and geographic scales in an endangered freshwater mussel *Unio durieui* (Bivalvia: Unionidae) endemic to northern Tunisia

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ABSTRACT

The freshwater mussel *Unio durieui* is a rare bivalve endemic to North African watersheds. Although this is an important species from an ecological and conservation perspective, information on its genetic diversity and population structure is lacking. Here, we combine geometric morphometric and molecular genetic analyses to characterize and evaluate the status of populations of this endangered species from four Tunisian watershed basins (Cap Bon, Ichkeul, Northwest and Mejerda River basins). By using a multiparameter approach, we sought to test the hypothesis that populations from these geographic regions could be considered as distinct management units. Shell outline shapes were assessed using elliptic Fourier analysis followed by linear discriminant analysis of principal components. Multivariate analysis applied on normalized Fourier coefficients showed highly significant differences in shell shape among the populations. Population genetic analysis based on inter-simple sequence repeats revealed low levels of diversity and high differentiation between the populations studied. Two groups of populations are clearly evident, thus separating samples from the Northwest from those from the other watersheds. Although there is no evidence for isolation by distance patterns, genetic and phenotypic diversity appeared significantly correlated with climatic variables. These findings suggest isolation by environment and the nominal species *U. durieui* may constitute two biological species. Conservation programme actions should consider this substructure in establishing effective recovery strategies for this endangered species.

INTRODUCTION

The characterization of genetic diversity is fundamentally important for conservation biology programmes because it helps determine the evolutionary context of endangered species (Avise, 1989). Analyses of genetic variation in a population genetics context can be used to reconstruct the evolutionary history, examine the contemporary status and predict the future of endangered species (Hedrick & Hurt, 2012). The simplest use of population genetics for conservation purposes is to identify conservation units (Moritz, 1994; Funk *et al.*, 2012; Coates, Byrne & Moritz, 2018) that are sufficiently disconnected to require separate protection measures. This goal can be attained with nonspecific molecular markers, which can be developed rapidly and at low cost for nonmodel species (Avise, 2004).

Inter-simple sequence repeats (ISSRs) are a type of molecular marker with three major advantages: their development does not require DNA sequence information, the relevant laboratory protocol is relatively quick and economical, and associated datasets may be characterized by substantial polymorphism (Zietkiewicz, Rafalski & Labuda, 1994). Morphological variation can sometimes be used as a proxy of genetic variation in study contexts where the possibility that morphology is subject to environmental plasticity can be ruled out. Establishing whether this condition is fulfilled requires studying both the morphological and genetic variation of the same specimens.

The rapid current decline in freshwater biodiversity has become a major concern in conservation worldwide (Ricciardi & Rasmussen, 1999; Lydeard *et al.*, 2004). Freshwater molluscs are among the most endangered groups of organisms, with much higher rates of decline than other animals (Lydeard *et al.*, 2004; Régnier, Fontaine & Bouchet, 2009). Climate change, habitat degradation, deterioration of water quality, introduction of nonnative species and

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Table 1. Collection data for *Unio durieui* specimens sampled from northern Tunisian rivers.

Watersheds	River names	Code	Collection date	Sample size	Shell length (mm ± SE)	Altitude (m)	Longitude	Latitude
Cap Bon	Abid River	AD	26 January 2012	7	58.42 ± 1.09	2.2	10.72469	36.86728
Ichkeul	Douimiss River	DM	25 April 2012	24	52.99 ± 1.15	7	9.62403	37.20008
Northwest	Barbra River	BR	21 May 2012	7	77.97 ± 1.88	117.1	8.56258	36.77492
	Kebir River	KB	25 April 2012	6	53.49 ± 4.34	8.5	8.75406	36.91920
	Maâden River	MA	7 July 2012	13	57.77 ± 1.48	86.9	9.10656	36.90600
Mejerda*	Mejerda River	MJ	21 May 2012	3	66.52 ± 5.62	170.7	8.54222	36.46936
	Maleh River	ML	21 May 2012	2	64.03 ± 4.90	173.7	8.57561	36.50047

*For analytical purposes, samples from the Mejerda watershed were combined into a single population under the code MJ.

overexploitation are the major causes of their decline (Bogan, 1993; Dudgeon *et al.*, 2006; Downing, Van Meter & Woolnough, 2010; Lopes-Lima *et al.*, 2018).

The freshwater mussels of the family Unionidae are widely distributed on all continents, except Antarctica (Graf & Cummings, 2021). This family forms the most species-rich group of freshwater bivalves, with 760 identified species represented by 155 genera being currently recognized (Graf & Cummings, 2021). Unionids are valuable components of the aquatic ecosystems in which they occur because they provide important ecosystem services, such as water filtration and sediment stabilization (Vaughn, 2018). Many unionids have undergone severe population declines (Bogan, 1993; Dudgeon *et al.*, 2006; Downing *et al.*, 2010; Lopes-Lima *et al.*, 2018), leading to decreases in genetic diversity due to processes, such as inbreeding depression and genetic drift, which lead to the loss of fitness and local adaptation (Frankham, Ballou & Briscoe, 2002; Reed *et al.*, 2003; Frankham, 2005). Understanding the population genetic structure of unionids can improve management of their genetic diversity.

In Tunisia, the species diversity of unionid mussels is very low, with only five species currently recognized (Khalloufi *et al.*, 2011). Three of them belong to the genus *Unio*: *Unio ravoisieri* (Deshayes, 1847), *U. durieui* (Deshayes, 1847) and *U. gibbus* (Spengler, 1793). While *U. ravoisieri* is endemic to North Africa, it is the most frequently encountered freshwater mussel species in this region (Khalloufi *et al.*, 2011; Fassatoui, Ben Rejeb Jenhani & Romdhane, 2015, 2019; Fassatoui & Romdhane, 2021). *Unio gibbus* has been assessed as belonging to the category 'Least Concern' in the International Union for the Conservation of Nature's (IUCN) Red List as this species has a very large distribution encompassing almost all of Europe and part of Asia. *Unio durieui*, in contrast, falls into IUCN 2010 Red List's 'Endangered' category (Van Damme *et al.*, 2010).

Apart from a preliminary taxonomic study of molecular and morphological characters of *U. durieui* in Tunisia (Khalloufi *et al.*, 2011), no study of the genetics of this species has been carried out to date. The geographic distribution of *U. durieui* in Tunisia is limited to the humid and subhumid bioclimatic regions of the country and extends over four watersheds in the north, namely the Cap Bon, Ichkeul, Northwest and Mejerda watersheds.

The objective of this study was to assess genetic and shell phenotypic variation in *U. durieui*, within and among four river drainages in northern Tunisia, which collectively cover the distributional range of this species. We used ISSR markers to measure genetic diversity and structure, and we performed an outline-based geometric morphometric analysis based on the means of elliptical Fourier descriptors to evaluate shell shape. We compared genetic and morphological variation among samples and used multiple approaches (i.e. redundancy analysis, Mantel testing and multiple matrix regression) to examine the relationship between geography and environment, on the one hand, and genetic and morphological diversity, on the other hand. Our findings will contribute to the development of future conservation strategies and actions for *U. durieui*.

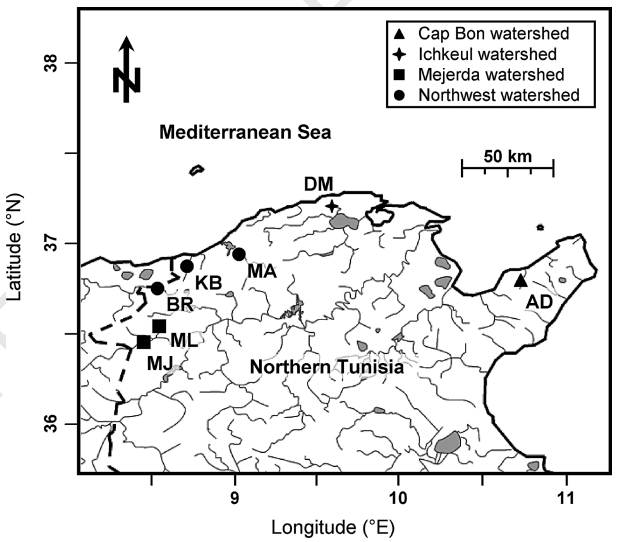


Figure 1. Map of northern Tunisian rivers showing collection sites of *Unio durieui*. Abbreviations: AD, Abid River; BR, Barbra River; DM, Douimiss River; KB, Kebir River; MA, Maâden River; MJ, Mejerda River; and ML, Maleh River. Sites belonging to the same watershed are indicated by the same symbol. Samples codes follow Table 1.

MATERIAL AND METHODS

Sample collection

Samples of *Unio durieui* were collected between January and July 2012 from the four main hydrological regions of northern Tunisia, that is the Cap Bon, Ichkeul, Northwest and Mejerda River basins. The sampling comprised a total of 62 individuals from seven rivers with sample sizes ranging from 2 to 24 specimens per sampled site (Table 1 and Fig. 1). The rivers within each watershed are generally not interconnected; the exception to this are the Mejerda and Maleh Rivers of the Mejerda Basin. For this reason and given our small sample sizes, the samples from the Mejerda watershed were combined into a single population to obtain a minimum of five individuals; this combined sampled is referred to the Mejerda population in what follows. Sampled freshwater mussels were identified to species level based on the external shell and colour, following Khalloufi *et al.* (2011). Shell length, defined here as the maximum distance between anterior and posterior shell margins, was determined to the nearest 0.01 mm with a digital calliper. Samples of foot tissue preserved in 95% ethanol were used for molecular systematic study. All sampled individuals were dissected and sex was determined by examination of gonad smears under a microscope (model: LEITZ DIALUX 20 EB). Following Heard (1975), mussels with oogonia, oocytes or larvae (glochidia) at any stage of development were sexed as females and mussels containing spermatozoa and/or sperm morulae were sexed as males. Given the small sample sizes, the two sexes were pooled for subsequent analyses.

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Table 2. Characteristics of the eight ISSR markers with the best resolution for *Unio durieui*.

Marker	Sequence no.	T_m (°C)	TNB	NPB	%*P*	PIC	RP	*H*′
UBC811	5′-$(GA)_8$C-3′	52	14	12	85.71	0.327	6.285	3.018
UBC814	5′-$(CT)_8$TG-3′	54	8	7	87.50	0.291	2.877	1.778
UBC834	5′-$(AG)_8$YT-3′	54	15	12	80.00	0.202	3.545	2.262
UBC843	5′-$(CT)_8$RA-3′	56	9	8	88.89	0.239	2.686	1.819
UBC844	5′-$(CT)_8$RC-3′	52	12	8	66.67	0.372	4.390	2.458
UBC845	5′-$(CT)_8$RG-3′	54	12	12	100	0.273	4.508	2.982
UBC874	5′-$(CCCT)_4$-3′	56	5	5	100	0.451	3.782	1.665
UBC898	5′-$(CA)_6$RY-3′	56	12	11	91.67	0.354	5.680	3.169
Average/primer				0.314	4.219	2.394		

Abbreviations: T_m, annealing temperature; TNB, total number of bands; NPB, number of polymorphic bands; %*P*, percentage of polymorphism; PIC, polymorphic information content; RP, resolving power; and *H*′, Shannon's index. The characters R and Y represent the nucleotide bases A or G and C or T, respectively.

Molecular analyses

DNA isolation and ISSR PCR amplification: Genomic DNA was extracted from small pieces of foot tissue (*c.* 20 mg) using a modified salting out protocol, as described by Sunnucks & Hales (1996). The DNA was amplified with PCR using ISSR primers from the University of British Columbia (UBC, Vancouver, Canada). Sixteen ISSR primers were screened for their repeatable amplification. Eight of these ISSR primers produced clear and reproducible bands, and were retained for the present study (Table 2). Each primer pair was amplified in a total volume of 25 μl, consisting of 2 µl genomic DNA, 2.5 μl 10× PCR buffer, 1 μl $MgCl_2$ (50 mmol/l), 2 μl dNTPs (1.25 mmol/l), 0.2 μmol/l primer, 1 U of Taq polymerase enzyme (Invitrogen) and sterile distilled water. PCR amplification was carried out in a DNA thermal cycler (Life Technology Applied Biosystems 2720 thermal cycler). Reaction conditions were as follows: initial denaturation for 5 min at 94 °C; 45 cycles of denaturation for 40 s at 94 °C, annealing for 40 s at the appropriate annealing temperature (see T_m values in Table 2) and extension at 72 °C for 1 min 30 s; and a final extension at 72 °C for 8 min. The amplified products (6 µl of PCR products mixed with 2 µl of bromophenol blue) were run on a 2% agarose gel and successful amplifications were selected. To ensure repeatability, all individuals were visually scored twice for all eight ISSR primers by independent readers.

Genetic data analyses: Each amplified ISSR locus was transformed into a binary character matrix (1 = presence, 0 = absence). The effectiveness of each primer was evaluated by the following parameters: the total number of bands (TNB), the number of polymorphic bands (NPB), the percentage of polymorphism (%*P*), the polymorphic information content (PIC) (Roldàn-Ruiz *et al.*, 2000), the resolving power (RP) (Prevost & Wilkinson, 1999) and the Shannon's index (*H*′) (Shannon & Weaver, 1949). PIC of dominant biallelic data was estimated by the formula $PIC = (\Sigma 2p_iq_i)/NPB$, where p_i is the frequency of the visual allele (band present) and q_i is the frequency of the null allele in each locus of the ISSR primer under consideration. Allele frequencies were estimated assuming the population is at the Hardy–Weinberg equilibrium for this locus, so q_i is the square root of frequency of individuals for which a band is not observed. RP is defined per primer as $RP = \Sigma IB_i$, where IB_i is the band informativeness; this takes the values of $1 - (2 \times |0.5 - p_i|)$, with p_i being the frequency of the visual allele in each locus of the relevant ISSR primer. Shannon's index was calculated with the formula $H' = -\Sigma p_i \ln p_i$. In calculating PIC, RP and the Shannon index, we used allele frequencies under the Hardy–Weinberg equilibrium.

The genetic diversity at the population level was estimated using the observed number of alleles per locus (n_a), the percentage of polymorphic loci (%*P*), the number of polymorphic loci (NPL), the expected heterozygosity for each population (H_{exp}) and the Shannon–Wiener diversity index (*H*′). In addition, we calculated the average gene diversity within population (H_S; i.e. mean of expected heterozygosity over all populations) and the total diversity (H_T; i.e. expected heterozygosity of all individuals in all samples pooled into a single population) assuming the Hardy–Weinberg equilibrium (Nei, 1987). Global genetic differentiation was also estimated by Nei's (1987) gene diversity statistic (G_{ST}). All these parameters were calculated using POPGENE v. 1.32 (Yeh, Yang & Boyle, 1999). Unless stated otherwise, all remaining analyses were carried out in the R environment (R Development Core Team, 2017). Discriminant analysis of principal components (DAPC) was performed to investigate the population structure of *U. durieui* based on allele frequencies of ISSR loci; for this, we used the R package adegenet v. 2.1.1 (Jombart, 2008). This method does not assume the Hardy–Weinberg or gametic equilibrium and does not make any assumptions on the cause of structure (i.e. island model *vs* isolation by distance). DAPC analysis was performed using the dapc function, and the optimal number of principal components (PCs) to retain was assessed using the optim.a.score function. Membership probabilities for each individual with respect to its population of origin were plotted using the compoplot function. Using the find.cluster function and following Jombart, Devillard & Balloux (2010), the number of genetic clusters was identified based on the Bayesian information criterion (BIC).

We also used the R package LEA v. 3.2.0 (Frichot & François, 2015) to assign individuals to genetic clusters. The most likely number of ancestral populations was determined with the Bayesian population assignment algorithm using the snmf function. The number of putative clusters (*K*) was allowed to range from 1 to 10, with ten repetitions of each for a maximum of 1,000 iterations. The minimum cross-entropy was used to decide the best value of *K*.

Two hierarchical analyses of molecular variance (AMOVAs) were computed using the R package poppr v. 2.8.1 (Kamvar, Tabima & Grünwald, 2014). The significance of the genetic structure was tested using 9,999 permutations. Variance components were extracted for three hierarchical levels: (1) among individuals within samples, (2) among samples within groups of samples and (3) among groups of samples. Groups of samples were defined according to the sampled watershed (Table 1) or following the outcomes of the DAPC. A minimal spanning network (MSN) was constructed based on Nei's genetic distance using poppr.

Shell shape analysis

Image and shell contour acquisition: Digital images of shells were obtained in standardized conditions to minimize distortion errors. Each left valve was placed on a dark background with the external side facing down, the umbo upwards and the valve positioned in a horizontal line; each valve was then digitized using a high-resolution video camera (model: Sony DSC-W610, 14.1 MP). The

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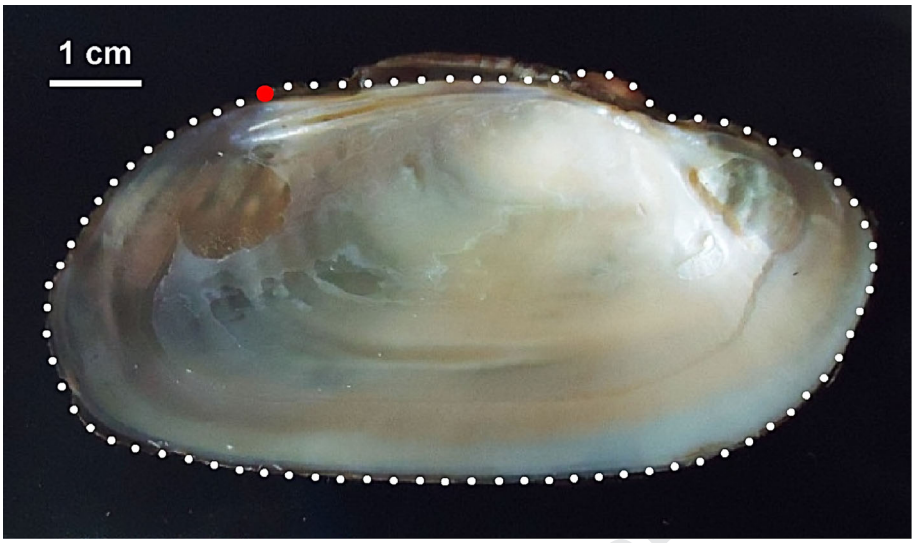


Figure 2. Left valve of *Unio durieui* showing the position of the sliding semi-landmarks (80 dots along the contour) used to evaluate the shape variability of the shells collected in seven rivers from northern Tunisia. In all specimens, the large red dot at the posterior end of the hinge ligament was the anchoring point for the semi-landmarks extending towards the lateral side of the valve.

magnification was kept constant for all the shells photographed. Digital images of each shell were stored in jpg format and were processed using tpsDig v. 2.17 (Rohlf, 2013). Because informative homologous points for landmark placement in bivalve shells are difficult to replicate, we treated shell shapes as curves and adopted the semi-landmark method. The curve was defined by 80 equidistantly spaced semi-landmark points placed along the shell outline of each individual. For all the specimens, we used the posterior end of the hinge ligament as the anchoring point from which the semi-landmarks begin and extend towards the lateral side (Fig. 2). The *x*–*y* coordinates of each point of the shape for all shells were then extracted and used as input data to be processed by the R package momocs v. 1.3.0 (Bonhomme *et al.*, 2014). Variation in shell shape was assessed as closed outlines using elliptical Fourier analysis (Giardina & Kuhl, 1977; Kuhl & Giardina, 1982).

Elliptical Fourier analysis: Elliptical Fourier analysis consists of describing the outline of a specific shape using several components (harmonics) and with an ellipse as the first approximation step (Lestrel, 1997). Each harmonic is characterized by four coefficients (a_n, b_n, c_n and d_n) resulting from the sine and the cosine part of the variation in the x and y coordinates. As the first seven harmonics reached 99% of the cumulative power (see Supplementary Material Fig. S1), the Fourier analysis indicated that shell shape could be summarized by eight harmonics resulting in 32 elliptical Fourier coefficients per individual. Each shell was normalized for size, rotation, translation and orientation, which caused the degeneration of the first three coefficients to constant values ($a_1 = 1$, $b_1 = c_1 = 0$); the last coefficient was included because it represents the eccentricity of the ellipse. Accordingly, each individual was represented by 29 normalized elliptical Fourier descriptors (NEFDs) and these were used to perform the multivariate statistical analyses.

Statistical analysis: Possible effect of site and sex on shell shape NEFDs was tested by using multivariate analysis of variances (MANOVA) with Hotelling–Lawley's trace test of significance. A principal component analysis (PCA) was computed from the variance–covariance matrix to summarize shape variation based on the elliptical Fourier coefficients for each shell of *U. durieui*. In order to explore how shell shape variation was related to an allometric pattern between size and shape, a Spearman's rank correlation test was applied between total shell length (accounts for size) and the first PC axis (describes shell shape). On the basis of PC scores summarizing at least 99% of the total variance, linear discriminant analysis (LDA) was performed to discriminate among *U. durieui* populations. The performance of the LDA was assessed with values of Wilks' λ ranging from 0 (total discrimination) to 1 (no discrimination). From LDA, a leave-one-out cross-validation procedure was carried out to compute correct classification percentages for each population. Morphological diversity within populations was estimated as the sum of variances disparity statistic, using the R package dispRity v. 1.2.3 (Guillerme, 2018); for this, we used all PC axes scores determined from elliptical Fourier coefficients. In order to correct for sample size, morphological disparity was calculated with rarefaction during the bootstrapping procedure. A significance level of 0.05 was set for all statistical tests used.

Geospatial and environmental data analyses

To examine the effect of latitude and longitude on individual genetic diversity and shell shape variation, we performed a redundancy analysis (Legendre & Legendre, 1998). For these analyses, our response variables were the first PC summarizing genetic diversity and the first PC summarizing shell shape variation and our explanatory variables were the decimal geocoordinates (latitude and longitude) for each individual. We performed this analysis using the rda function of the R package vegan v. 2.5.3 (Oksanen *et al.*, 2018). The effect of latitude and longitude was tested separately (based on a permutation test of 9,999 iterations) by the anova.cca function of the vegan package.

At the population level, the patterns of isolation by distance (IBD) and isolation by environment (IBE) were tested using the Mantel procedure in vegan. The geographic distances as well as environmental distances between populations were regressed against Nei's genetic distances and squared Mahalanobis distances of the shell shape. The significance of the correlation between distance matrices was assessed using 9,999 permutations.

We also used multiple matrix regression with randomization (MMRR) (Wang, 2013) as an alternative method to Mantel tests, with spatial distances (β_D) and environmental distances (β_E) being simultaneously regressed against genetic distances and shell shape distances, separately. Analyses were performed using the R function MMRR (Wang, 2013), with 9,999 permutations.

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Table 3. Details of genetic diversity per sample in *Unio durieui*.

Sample code	n_a	NPL	%*P*	H_{exp}	H'	H_S	H_T
AD	1.437	38	43.68	0.168	0.246		
BR	1.414	36	41.38	0.136	0.207		
DM	1.563	49	56.32	0.151	0.234		
KB	1.333	29	33.33	0.117	0.176		
MA	1.494	43	49.43	0.153	0.234		
MJ	1.322	28	32.18	0.108	0.164		
Total	1.862	75	42.91	0.139	0.396	0.139	0.269

Abbreviations: n_a, mean observed number of alleles per locus; NPL, number of polymorphic loci; %*P*, percentage of polymorphic loci; H_{exp}, expected heterozygosity; H', Shannon's index; H_S, genetic diversity within population; and H_T, total genetic diversity according to Nei (1987).
Sample codes follow Table 1.

Squared Mahalanobis distances of shell shape were determined for all elliptical Fourier coefficients. Geographic distances were calculated in km as horizontal straight-line distances between localities using Google Earth Pro v. 7.3.4.8248 (www.google.com/intl/en/earth/versions/#earth-pro). For environmental distances, seven climatic variables that characterize each sample site were determined: altitude, monthly mean minimum temperature, monthly mean maximum temperature, annual rainfall height, annual number of rainy days, annual number of sunny days and Emberger's pluviothermic quotient (Emberger, 1930). Climatic variables were obtained from National Institute of Meteorology of the Tunisian Republic (http://www.meteo.tn/en) and were based on data from those weather stations that were closest to the sampling sites. These data correspond to climate means for the last 30 years. A PCA was performed on these climatic variables and this clustered samples according to their respective habitats. Finally, squared Mahalanobis distances between populations were calculated for the first PC axes that explain more than 95% of the total variance.

RESULTS

Genetic diversity, population structure and relationships

The eight ISSR primers employed revealed the presence of a total of 87 loci, from which 75 are polymorphic. TNB ranged from 5 to 15 and product sizes ranged from 350 to 2,500 bp. Table 2 summarizes the data obtained for the eight ISSR primers. The mean value of H' was 2.394 ± 0.570 (SD) for all primers; the lowest value was observed for UBC874 (1.665) and the maximum for UBC898 (3.169).

Low genetic variation was observed in all samples of *Unio durieui* with an average percentage of polymorphic loci (%*P*) of 42.91%. H' at the population level was 0.396 ± 0.248. H_S and H_T were 0.139 ± 0.012 and 0.269 ± 0.034, respectively. Details of genetic diversity per population are provided in Table 3. Some of the bands (4.6%) were identified as private bands (i.e. observed in a single population) across all samples (15 loci). The highest numbers of private bands were observed in Maâden (9) followed by Abid (4) and then Douimiss (2). The mean value of G_{ST} was 0.483, indicating a high level of population differentiation.

The DAPC used six PCs (see Supplementary Material Fig. S2A) and two discriminant functions (Fig. 3A), and the proportion of conserved variance was 55.6%. The BIC curve in the DAPC indicated $K = 2$ as the best clustering option for our data (see Supplementary Material Fig. S2B). DAPC separated individuals into two groups along the first axis with no overlap of 95% inertia ellipses. The two groups were the Northwest watershed samples and the group uniting Ichkeul, Mejerda and Cap Bon watersheds (see Table 1). Individual membership probabilities in relation to their populations of origin are shown in Fig. 3B. The LEA analysis supported the results of the DAPC. The Bayesian population assignment algorithm gives a minimum cross-entropy value of $K = 2$, suggesting, however, the existence of two ancestral clusters for *U. durieui* (Fig. 4A). Individual admixture proportions for each cluster are displayed in Fig. 4B.

Hierarchical analysis of genetic variation using AMOVA (Table 4) revealed highly significant differentiation both among watersheds and among populations within watersheds ($P < 0.001$). The partitioning genetic variance revealed that variance was higher within samples (51.4%) than among samples within groups (31.5%) and among groups (17.1%) based on the watershed grouping; patterns were similar when the AMOVA was based on groups defined in the DAPC.

The unbiased Nei's genetic distance among samples ranged from 0.097 to 0.307, with an average of 0.190 ± 0.058. The smallest distance was found between samples from the Kebir and Maâden Rivers, while the largest was between samples from the Barbra and Mejerda Rivers (Table 5). Cluster analysis based on Nei's genetic distance, using the MSN method, showed that reflecting patterns of geographical proximity, the *U. durieui* samples are relatively interconnected (see Supplementary Material Fig. S3).

Geometric morphometrics of shell shape

The MANOVA of the NEFDs (Table 6) confirmed that shell shape variation was highly significant among populations (Hotelling–Lawley's trace = 44, approximate $F_{3,87} = 4$, $P = 0.00024$). However, we found that sex had no effect on shell shape variation (Hotelling–Lawley's trace = 3, approximate $F_{3,29} = 1$, $P = 0.545$).

PCAs of the elliptical Fourier coefficients indicated that over 99% of the observed outline shape variation can be represented on ten orthogonal PC axes. The first two PC axes explained 86.5% of the overall variance in shell shape (Fig. 5). Populations were separated mainly along the first axis, even though a strong overlap was observed. Spearman's rank correlation of size and shape showed a strong allometric relationship in *U. durieui* (see Supplementary Material Fig. S4). We found a highly significant negative correlation between total shell length and shell shape ($r_S = -0.781$, $P < 0.0001$), indicating that the more elongated elliptical shell shape of older individuals was larger than the oval rounded shape of younger individuals.

The results of the LDA of shell shape showed that the first two axes account for more than 85% of the total variance (Fig. 6). The LDA returned the highest correct classifications, successfully discriminating among samples ($\lambda = 0.0128$, approximate $F_{5,80} = 6.958$, $P < 0.0001$). Overall, the discriminant analysis with cross-validation procedure correctly classified 77.41% in combined samples, but there was strong variation among populations: 95.83% for Douimiss, 84.61% for Maâden, 71.43% for Abid, 60.00% for Mejerda, 57.14% for Barbra and 33.33% for Kebir.

The sum of variances disparity for shell shape shows some variation between localities (see Supplementary Material Fig. S5). The Douimiss population displayed the highest disparity, followed by the Mejerda and Kebir populations (despite their low sample size), revealing that these populations exhibit substantial morphological diversity relative to the other populations. The smallest mean disparity is seen in the Barbra population.

Effect of geospatial and environmental factors on genetic and shell shape variation

Redundancy analyses performed to determine the relative contribution of geospatial components (latitude and longitude) to our response variables (either genetic or shell shape patterns) revealed significant effects (Table 7). Latitude and longitude explained a

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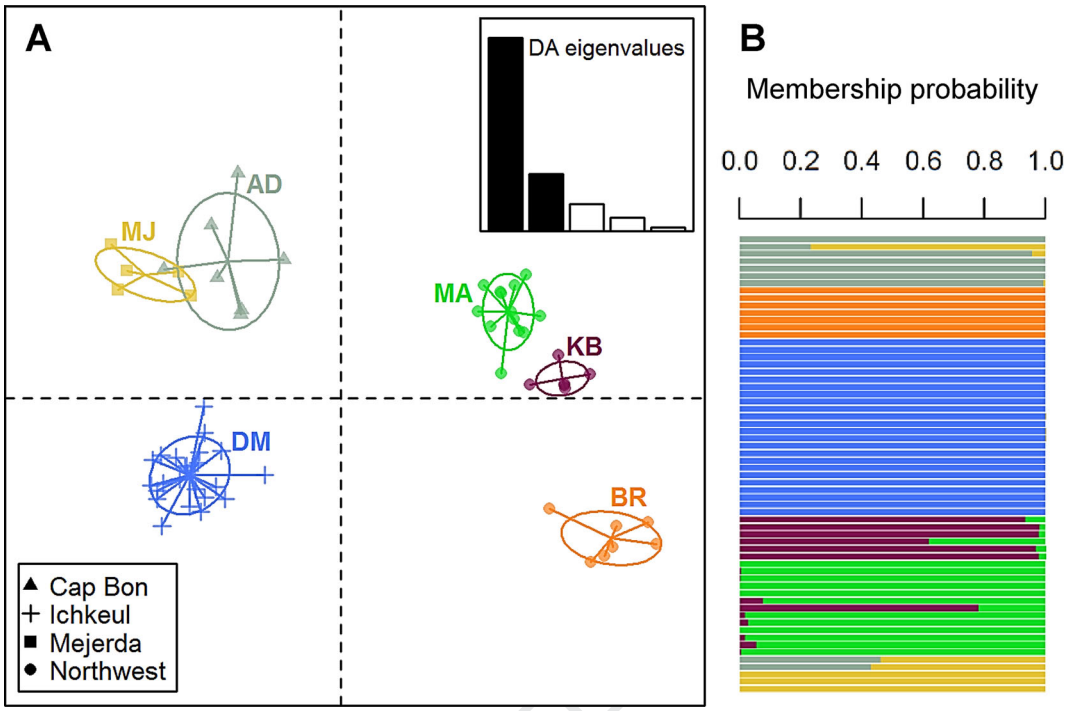


Figure 3. A. DAPC plot of the first two axes based on the eight ISSR primers. **B.** Bar plot showing accession membership probability of individuals in relation to their populations of origin. Ellipses represent 95% confidence intervals around the centroid of the distribution of each sample. Individuals from a single watershed are indicated by the same symbol. Samples codes follow Table 1.

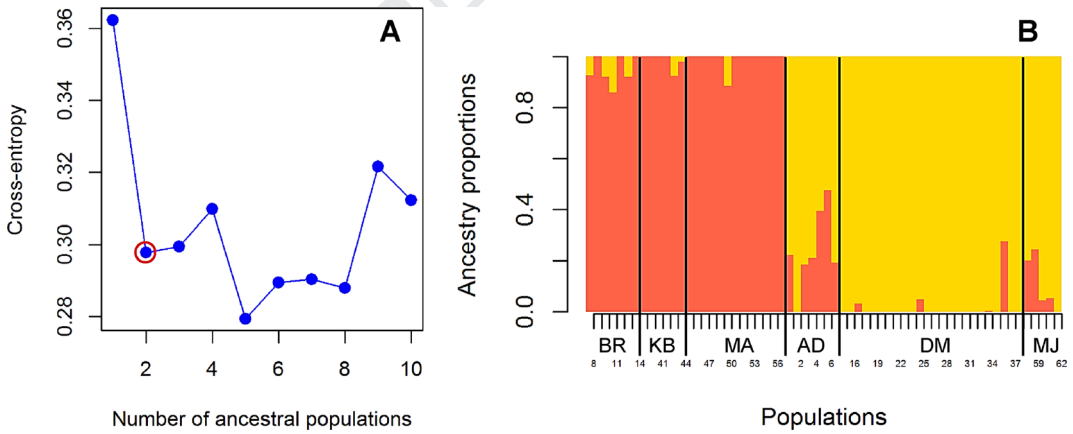


Figure 4. Estimated population structure for *Unio durieui* from northern Tunisian rivers as inferred by the snmf function of the LEA package. **A.** The cross-entropy criterion as a function of the number of populations showing the best value for $K = 2$ (surrounded by the red circle). **B.** Plot of population structure showing differentiation between two main genetic groups (shown in different colours). Sample codes follow Table 1.

similar proportion of the variance in genetic variation (26.44% and 27.75%, respectively), indicating that both of these explanatory variables have a similar effect on genetic diversity. When we controlled for the effects of geospatial variables on shell shape, we found longitude to be a stronger predictor of shell shape variation than latitude; longitude explained a greater proportion of the variance (41.40%) than latitude (11.68%).

The PCA of the climatic variables showed that the first PCs explain more than 95% of total variance in the dataset (PC1 = 54.83%, PC2 = 32.23%, PC3 = 9.76%), of which only the first two have eigenvalues >1 (see Supplementary Material Fig. S6). These results can be used as a proxy to quantify environment heterogeneity among sites and show that samples from Cap Bon, Ichkeul and Mejerda watersheds are separated mainly by temperature, whereas samples from the Northwest watershed are separated mainly by rainfall.

Mantel tests showed that spatial distances were correlated neither with unbiased Nei's genetic distances ($r = -0.254$, $P = 0.775$) nor with Mahalanobis distances of shell shape ($r = -0.380$, $P = 0.783$). Environmental distances did not carry significant additional information about genetic distances ($r = 0.337$, $P = 0.125$) but revealed a low significant correlation ($r = 0.615$, $P = 0.042$), suggesting that IBE may explain the pattern of morphological variation. The relationship between genetic distances and Mahalanobis distances of shell shape was not significant ($r = -0.004$, $P = 0.467$).

The MMRR analysis showed that environmental distances correlated significantly with genetic distances ($\beta_E = 0.00386$, $P = 0.0037$), whereas spatial distances did not correlate significantly

Table 4. AMOVA based on ISSR markers of six samples of *Unio durieui*.

	df	Sum of squares	Variance components	Percentage of variance	Φ-statistics	*P*-value
Samples grouped by watersheds						
Within samples	56	403.893	7.212	51.433	0.486	<0.001
Among samples within groups	2	86.163	4.420	31.520	0.380	<0.001
Among groups	3	256.573	2.390	17.047	0.170	<0.001
Total	61	746.630	14.023	100		
Groups defined by DAPC						
Within samples	56	403.893	7.212	48.904	0.511	<0.001
Among samples within groups	4	185.870	4.594	31.154	0.389	<0.001
Among groups	1	156.866	2.941	19.942	0.199	<0.001
Total	61	746.629	14.748	100		

Samples are separated into four groups according to the sampled watersheds (see Table 1) and into two groups, as defined by the DAPC (group 1: AD, DM and MJ; group 2: BR, KB and MA; see Table 1 for sample codes).

Table 5. Pairwise Nei's unbiased genetic distances between samples of *Unio durieui*.

	AD	BR	DM	KB	MA
BR	0.2539				
DM	0.1360	0.2010			
KB	0.1791	0.1506	0.2108		
MA	0.2317	0.1091	0.2091	0.0974	
MJ	0.1531	0.3075	0.1332	0.2503	0.2353

Sample codes follow Table 1.

Table 6. Summary statistics for MANOVA of shell shape normalized using elliptical Fourier descriptors.

	df	*T*	Approximate *F*	Num df	Den df	*P*
Intercept	1	196,202	60,890	29	9	<0.001
Population	3	44	4	4	87	<0.001
Sex	1	3	1	1	29	0.545

Test statistics: *T*, Hotelling–Lawley test statistic; num df, number of degrees of freedom in the model; den df, number of degrees of freedom associated with the model errors.

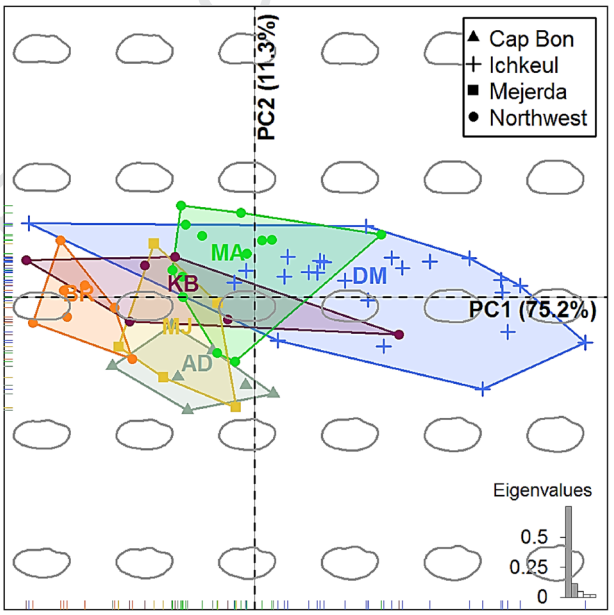


Figure 5. PCA plot of the first two PC axes based on elliptical Fourier coefficients between 62 individuals of *Unio durieui* from northern Tunisian rivers. The morphospace plotted over the observations represents theoretical shapes reconstructed based on the PCA scores. Individuals from a single watershed are indicated by the same symbol. Sample codes follow Table 1.

with genetic distances ($\beta_D = -0.00032$, $P = 0.11$). Both factors together explained nearly 32% of the observed genetic variability ($R^2 = 0.322$, $P = 0.027$) (Fig. 7A). Similarly, the relationship between shell shape and environmental distances was significant ($\beta_E = 2{,}581{,}695.4$, $P = 0.009$), but that between shell shape and geographic distances was not significant ($\beta_D = -274{,}264.1$, $P = 0.067$). The combined effect of both factors accounted for more than 75% of the observed shell variability ($R^2 = 0.753$, $P = 0.009$) (Fig. 7B).

DISCUSSION

Genetic diversity in a population is directly related to its effective size (i.e. resulting from various factors, such as breeding system and gene flow) and is an indicator of the potential to adapt to future environmental change (Frankham *et al.*, 2002; Reed *et al.*, 2003; Frankham, 2005). Many endangered species display characteristically low genetic variation (Spielman, Brook & Frankham, 2004). We found that Tunisian *Unio durieui* populations are characterized by low levels of genetic diversity in ISSR markers, with 42.91% of bands showing polymorphism and about 14% of individuals being heterozygous. Our values for heterozygosity appear relatively lower than those observed in the unionid freshwater mussel *Hyriopsis cumingii*, for which average heterozygosities have been shown to range from 0.47 to 0.20 for the same set of genetic markers as used in the present study (Li, Wang & Bai, 2009). Using other genetic markers, including mtDNA and microsatellites, low genetic diversity has also been reported for several endangered freshwater mussels (e.g. Zanatta & Murphy, 2008; Lane, Hallerman & Jones, 2019; Menon *et al.*, 2019; Walters, Taynor & Berg, 2021).

Genetic and shell shape data revealed a high degree of differentiation between *U. durieui* populations, suggesting independent evolutionary histories for and low gene flow between these populations, as well as high levels of genetic structure even within each watershed. Two groups of populations are particularly well distinguished and they also correspond to distinct environmental conditions: the Northwest group of samples, including those from the Barbra, Kebir and Maâden Rivers, and the other samples, notably Abid, Douimiss, Mejerda and Maleh Rivers, which form a separate group.

Gene flow plays a critical role in differentiation among populations (Slatkin, 1987). The remarkable degree of differentiation in ISSR markers may reflect the existence of poor connectivity among the sampled populations of *U. durieui*. Indeed, river ecosystems are often considered as complex environments greatly influencing gene

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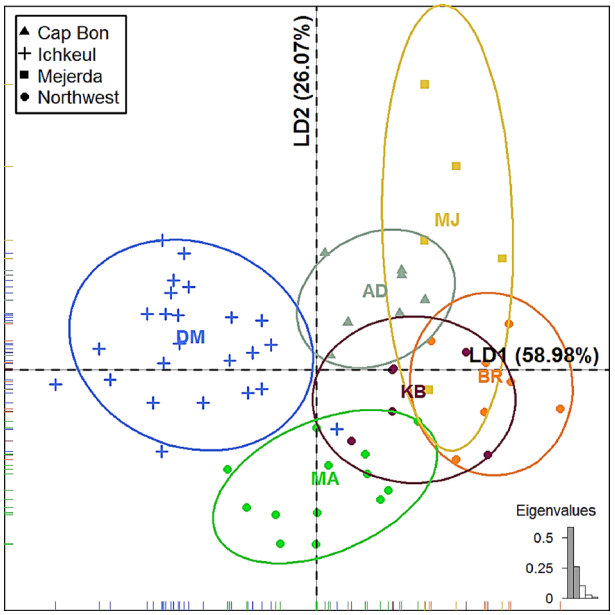


Figure 6. LDA plot (based on normalized elliptical Fourier descriptors) of the first two functions for shell outlines of *Unio durieui* from northern Tunisian rivers. Ellipses represent 95% confidence intervals around the centroid of the distribution of each sample. Individuals from a single watershed are indicated by the same symbol. Samples codes follow Table 1.

Table 7. Redundancy analysis models examining the effect of latitude and longitude on genetic and shell shape variations in *Unio durieui*.

Variable	Total variance	%Variation	df	*F*	*P*
Genetic variation					
Latitude	0.73182	26.44	60	21.569	<0.001
Longitude	0.76788	27.75	60	23.040	<0.001
Shell shape variation					
Latitude	0.00008	11.68	60	7.938	0.0061
Longitude	0.00030	41.40	60	42.393	<0.001

flow due to the hierarchical properties inherent in drainage networks, unidirectional water flow and relatively limited lateral connectivity (Davis *et al.*, 2018). Moreover, freshwater mussels, which tend to be less abundant in these ecosystems, have low dispersal capabilities due to their sedentary lifestyle and a complex reproductive cycle involving an obligate parasitic larval stage (glochidia) that is dependent on a host fish (Modesto *et al.*, 2018). Like all unionids, the mode of reproduction in *U. durieui* is gonochoric, with fertilization occurring without copulation and females being viviparous (Barnhart, Haag & Roston, 2008; Modesto *et al.*, 2018). The host of the parasitic stage of the glochidium is the barb *Barbus callensis*, which is endemic to North Africa and known as common teleostean host fish for mussel glochidia in this region; this fish is restricted to freshwater habitats and tolerates low salinity levels (Kraiem & Pattee, 1988).

Our analyses revealed no evidence of IBD (i.e. a correlation between genetic and geographic distances), a phenomenon that is often invoked to support the hypothesis that neutral processes may be responsible for genetic differentiation (Slatkin, 1993), as well as the correlation between shell shape and geographic distance. The lack of a correlation between genetic and geographic distances in our study contrasts with the morphometric study performed for eight samples of a closely related species, *U. ravoisieri*, which was collected from the same geographical region in northern Tunisia (Fassatoui *et al.*, 2015). Although the phenotypic differences in shell morphology were highly significant among samples, evidence for IBD pattern was found.

Conversely, we found a correlation between genetic and environmental distance, and thus evidence for IBE, and observed a correlation between shell shape and climatic distance. Although a Mantel test was nonsignificant for the first relationship, the MMRR analysis suggested a significant correlation between both response variables and environment distance. The combined effects of geographic and environment distances on our response variables were also significant as determined by the MMRR analysis. The mechanisms underlying IBD and IBE are fundamentally different (Lee & Mitchell-Olds, 2011; Wang, 2013; Wang & Bradburd, 2014). The former is based on physical barriers and distance (involving genetic drift and movement of individuals), whereas the latter is based on divergent natural selection and is usually related to adaptability to environmental pressure (Lee & Mitchell-Olds, 2011; Wang, 2013; Wang & Bradburd, 2014).

Our data are consistent with the occurrence of IBE in *U. durieui*, suggesting a significant effect of environmental factors on population adaptation, with climatic variables, particularly temperature and rainfall, being the abiotic factors that contribute most to this differentiation. The effect of selection pressure is generally faster than that of genetic drift and could occur at a small geographic scale. This main mediating force thus acts to link genes to climatic extremes. For example, the study by Manthey & Moyle (2015) found 79 loci putatively under selection in *Sitta carolinensis*; some of these loci were strongly associated with climate extremes, separating signals of IBE from IBD. Other published studies have reported evidence of IBE for various taxa (e.g. Byars, Parsons & Hoffmann, 2009; Barker *et al.*, 2011; Papadopulos *et al.*, 2014; Manthey & Moyle, 2015; Wu *et al.*, 2016; Polato *et al.*, 2017; Ruppert *et al.*, 2017). Our results thus strongly suggest that the populations of *U. durieui* studied are differentially adapted to distinct environmental conditions.

Generally, a morphogenetic correlation is expected if both traits are neutral. When shell shape is plastic enough, it can cause the lack of correlation. When the shell is subjected to selection by the environment, this can also alter morphogenetic correlations and the phenotype may be locally adapted (Nicolaus & Edelaar, 2018). In our case, it is not surprising because the spatial genetic structuring identified using ISSR data does not correspond with groupings based on shell contour morphology. Published studies on marine and freshwater molluscs have provided support for the plastic nature of shell morphology. This has been explained either by a lack of a correlation between population genetic structure and morphological variation (Hoffman *et al.*, 2010; Zieritz *et al.*, 2010; De Noia *et al.*, 2020) or on the basis of the results of reciprocal transplantation experiments (Moore, 1934; Hinch, Bailey & Green, 1986), which have demonstrated an important role for environmental heterogeneity in the variation of shell growth. Several hypotheses may be advanced to explain our findings: (1) the ISSR markers we have used are not in linkage disequilibrium with the genes responsible for shell shape traits; (2) morphological traits are controlled by relatively few genes and the differences in shell shape are of relatively recent origin; and (3) the most likely explanation, a nonsignificant correlation could be the result of nongenetic factors and thus not the result of evolutionary forces acting on shell shape. If environmental heterogeneity has a strong impact on shell shape diversity, differences between populations may be due to morphological plasticity and local adaptation (Edelaar, Jovani & Gomez-Mestre, 2017).

On the basis of our results, we can hypothesize that there could be two distinct morphologically cryptic species that are adapted to two different climatic environments. Cryptic species may have different environmental preferences, with changing environmental conditions affecting the fitness of individual species and the interactions between them in different ways (Chenuil *et al.*, 2019). The genetic differences found between our samples are strong enough to

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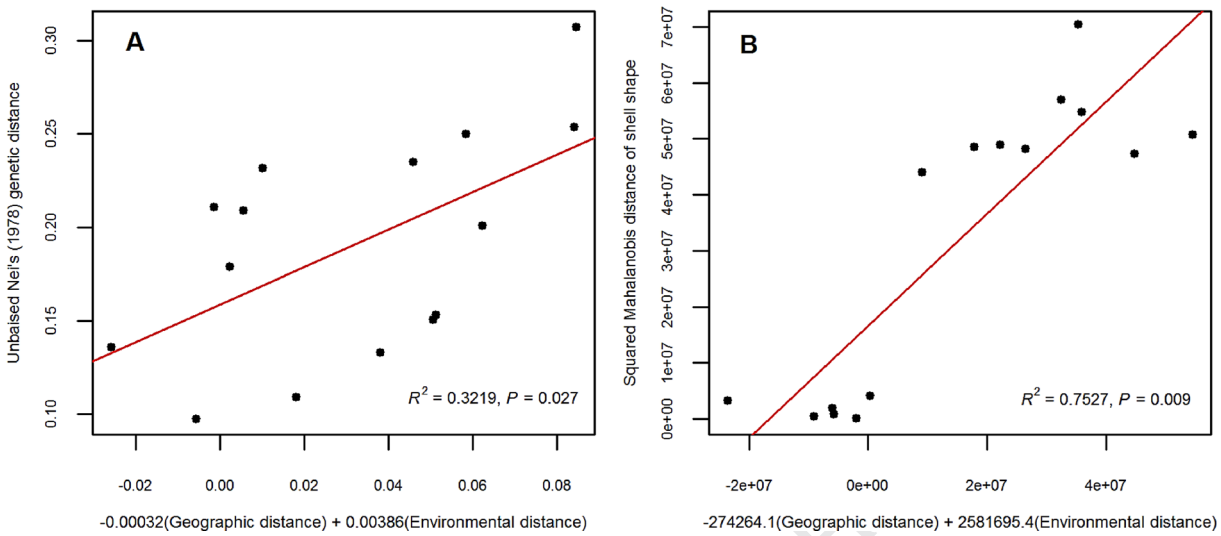


Figure 7. Scatterplots for the MMRR analysis, displaying the combined effects of both geographical and environmental factors on genetic distance (**A**) and shell shape distances (**B**) in populations of *Unio durieui* from northern Tunisian rivers.

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suggest that these different groups may be distinct cryptic species. This remains difficult to prove in the absence of cross-inheritance tests because the populations studied are allopatric. Nevertheless, the use of type II molecular markers is necessary to confirm this hypothesis.

The current study is the first to combine analyses of the molecular and morphological diversity with geospatial and environment data for the endangered freshwater mussel *U. durieui* from northern Tunisia. Planning of conservation management measures requires decisions about which sites will be selected for conservation and what measures will be taken at each of these sites. Moreover, identifying population structures as well as factors that promote population differentiation is essential for setting initial conservation priorities. According to Moritz (1994), there are two types of conservation units: the evolutionarily significant unit (ESU), which is concerned with historical population structure, phylogeny and long-term conservation needs, and the management unit, which addresses current population structure, allele frequencies and short-term management issues. The genetic differentiation detected for *U. durieui*, notably that between the Northwest watershed and the remaining areas, indicates that this endangered species consists of at least two distinct genetic groups. These results suggest that two distinct ESUs have to be considered for conservation and that the extinction risk of each of these ESUs must be evaluated separately. The potential association of genetic and shell shape variables with climatic factors indicates a possible role for selection and local adaptation (e.g. Barker *et al.*, 2011; Dowle *et al.*, 2015; Wu *et al.*, 2016). Accordingly, any future management aimed at ensuring the viability of *U. durieui* must as a first step ensure that as large as possible an area of the natural habitat of this species should be conserved. Future studies need to pursue a better understanding of demographic, genetic, environmental and deterministic factors, as well as of the interactions between these factors, as it is these factors and interaction that may affect the long-term survival of *U. durieui*.

SUPPLEMENTARY MATERIAL

Q4 Supplementary material is available at *Journal of Molluscan Studies* online.

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