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Population genetic structure of the endangered yellow spotted mountain newt (*Neurergus derjugini*: Amphibia, Caudata) inferred from mitochondrial DNA sequences

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The yellow spotted mountain newt (*Neurergus derjugini*) is a critically endangered species restricted to fragmented habitats in highland streams of the middle Zagros Mountain in Iran and Iraq. We examined the species phylogeography by investigating sequences of a mitochondrial fragment of the ND2 gene for 77 individuals from 15 locations throughout the species known distribution. We found relatively high haplotype diversity (0.82 ± 0.025) but low nucleotide diversity (0.0038 ± 0.00022) across all populations. Phylogenetic trees supported monophyly, and the segregation of haplotypes was concordant with the haplotype network. We found a significant correlation between geographical and genetic distances among populations ($r = 0.54$, $P < 0.01$), suggesting restricted gene flow. Molecular dating suggested that haplogroups diverged during the early or middle Pleistocene. Bayesian skyline plot provided evidence for an expansion of populations during the Pleistocene-Holocene transition period. Taken together, isolation by distance due to low dispersal capability, habitat fragmentation, and historical factors have shaped the current population structure of *N. derjugini*.

Key words: phylogeography, endangered species, demography, evolutionary history, climate oscillation, conservation.

INTRODUCTION

Environmental and geographic heterogeneity are significant factors contributing to spatial genetic diversity (Manel et al., 2003; Palo et al., 2003). In addition, genetic differentiation among populations can be interpreted as the result of historical evolutionary processes such as genetic drift, founder effects, and acclimatisation to past ecological conditions including climatic oscillations during the Pleistocene glacial cycles (Hewitt, 2000; Weese et al., 2012). Physical and geographical barriers that separate populations may reduce population connectivity and gene flow, and, as a result, populations diverge due to natural selection and random genetic drift (Zhang et al., 2016). Isolation-by-distance (IBD), the correlation of genetic divergence and geographic distances, is further inversely liked to effective population size (Sexton et al., 2014). Population divergence can also occur in different environments with evolving reproductive isolation due to ecologically-based divergent selection (Dyer et al., 2010; Freeland et al., 2010; Wang et al., 2013), a process termed isolation by ecology or isolation by environment (IBE; Zellmer et al., 2012; Shafer & Wolf, 2013).

Population genetic divergence originating from geographical or environmental factors can be demonstrated through correlations of genetic distance measures with geographical or environmental distances (Wang et al., 2013). Assessing casual relationships between environmental and geographic factors and the genetic structure of populations is difficult (Balkenhol et al., 2009) because the interactions among various factors cannot always be detected by isolation-by-distance alone (Kittlein & Gaggiotti, 2008). An integration of genetic and environmental data has been employed for many different goals, including the exploration of population genetic structure (Mota-Vargas & Rojas-Soto, 2012), selecting re-introduction sites (Martinez-Meyer et al., 2006), mapping the habitat of threatened species (Chunco et al., 2013), and designing appropriate management plans and conservation strategies (Gebremedhin et al., 2009). However, the ecological and geographical data which are necessary for devising the species conservation action plan are as yet lacking for many species (Farasat et al., 2016).

Neurergus derjugini (Nesterov, 1916) is a urodele species confined to living in highland streams of the mid-Zagros Mountains (630 and 2,057 masl), and a distribution range covering western Iran and parts of eastern Iraq. Inhabited streams are surrounded by open oak forest and other plants such as amygdales scrublands, deciduous dwarf-scrublands, and cushion shrub land. In the northern part of its distribution, *N. derjugini* can live in streams without natural vegetation cover, including flooding meadows, agricultural lands, rangelands and orchards (Afroosheh et al., 2016). However, drought, the collection of *N. derjugini* for the national and international pet trade, and habitat degradation are threats for this

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species. Fragmented habitats, the diminished number of subpopulations, a small area (< 10 km^2) for occupancy and a continuing decline in the range and quality of habitats are factors for listing *N. derjugini* as a Critically Endangered species by the International Union for Conservation of Nature (IUCN; Red List criteria: A3cde+4cde; B2ab [iii, iv, v] ver. 3.1) (Sharifi et al., 2009).

Here, we test the current genetic and geographical structure among populations of *N. derjugini* throughout the known distribution range in Iran and Iraq, based on partial sequences of the mtDNA ND2 genes, 1) to identify associations between genetic diversity and ecological-geographical differences, 2) to identify links between population genetics and climatic oscillations in the Quaternary and 3) to estimate levels of genetic variation within and among different populations of *N. derjugini*, in order to provide management plans for future conservation.

METHODS

Population sampling and sequencing

Population sampling was conducted for 15 populations throughout the range of species distribution in Iran and Iraq, via 22 sampling occasions during 2012 - 2014 (Table 1, Fig. 1a). Tissue samples were obtained from 77 individuals by removing a small section of the tail tip or toe using sterilised scissors. All tissue samples were stored in 95 % ethanol immediately after removal and then frozen at -20˚ C until processing. Genomic DNA was extracted using the GenNetBioTM tissue kit (Seoul, South Korea) following the manufacturer's instructions. A mitochondrial fragment of ND2 gene (1036pb) was amplified and sequenced using primer L3780, 5' TCG AAC CTA CCC TGA GGA GAT and H5018, 5' TCT GGG TTG CAT TCA GAA GA (Babik et al., 2005). PCR conditions used for this region consisted of an initial denaturation step at 94˚ C for 3 minutes, followed by 35 cycles of 30s denaturation at 94˚ C, 30 s annealing at 58˚ C and 60 s extension at 72˚ C, and a final extension step of 4 min at 72˚ C. Sequencing was performed by Macrogen Korea Laboratories. All sequences of partial mitochondrial ND2 have been deposited in the GenBank databases (accession numbers MK035716- MK035726 for Haplotypes 1-11).

Nucleotide polymorphisms

DNA sequences were aligned using Clustal W in the BioEdit v.7.0.5.3 (Hall, 1999) and by Muscle in MEGA 6 (Tamura et al., 2013). Five closely related taxa, *N. crocatus*, *N. kaiseri*, *N. strauchii*, *Triturus karelinii*, and *Ommatotriton vittatus* were used as outgroups, using existing GenBank under accession numbers (DQ517788, DQ517789, DQ517790, DQ517837 and, DQ517844). As an additional outgroup, ND2 of *N. kaiseri* was sequenced in the present study as described above. The number of haplotypes, polymorphic sites, parsimony informative sites, haplotype diversity (Hd) and nucleotide diversity (pi) were determined using DnaSP v 5.10.01 (Rozas et al., 2010) and Arlequin v 3.1 (Excoffier et al., 2005). Pairwise sequence divergence between haplotypes was calculated using the Kimura 2-parameter (K2P) model (Kimura, 1980) using MEGA 6 (Tamura et al., 2013), with standard errors calculated for 1000 bootstrap replicates.

Phylogenetic analyses

Phylogenetic relationships among haplotypes were determined by Bayesian analysis in MrBayes v3.2.2 (Ronquist et al., 2012) with 10,000,000 generations, sampling each 1000th generation, and Maximum likelihood (ML) analyses conducted in PhyML, v 3.0 (Guindon et al., 2010) with 1500 bootstrap replicates. jModelTest v 0.1.1 (Posada, 2008) was used to determine the best-fit substitution model for BI and ML analysis using the with Akaike information Criterion (AIK), and GTR+I

Table 1. List of sampling locations used in this study and haplotypes with genetic diversities and frequencies. SS: sample sizes, H: haplotypes and *Pi*: nucleotide diversity and *Hd*: haplotype diversity.

	Locality	Latitude	Longitude	Elev. (m)	Haplotypes and their frequencies	SS	H	*Pi*	*Hd*
1	Kavat	34º 52' N	46º 30' E	1601	Hap1(5), Hap2(1)	6	2	0.00032	0.333
2	Ghori ghale	34º 52' N	46º 29' E	1600	Hap1(5)	5	1	0.00000	0.000
3	Gholani	34º 54' N	46º 27' E	1575	Hap1(5)	5	1	0.00000	0.000
4	Dourisan	35º 01' N	46º 23' E	1600	Hap1(5)	5	1	0.00000	0.000
5	Darrenajjar	35º 05' N	46º 18' E	1472	Hap1(5)	5	1	0.00000	0.000
6	Lashkargah	35º 00' N	46º 08' E	1415	Hap3(1), Hap4(4)	5	2	0.00039	0.400
7	Nowdeshe	35º 11' N	46º 14' E	1760	Hap5(3), Hap6(2)	5	2	0.00174	0.600
8	Hani garmale	35º 14' N	46º 08' E	1383	Hap6(4)	4	1	0.00000	0.000
9	Tawale	35º 11' N	46º 11' E	1400	Hap6(4)	4	1	0.00000	0.000
10	Balkha	35º 12' N	46º 09' E	1482	Hap6(4)	4	1	0.00000	0.000
11	Penjwin	35º 36' N	45º 58' E	1421	Hap7(4), Hap8(1)	5	2	0.00039	0.40
12	Siya gwez	35º 47' N	45º 47' E	1689	Hap7(6)	6	1	0.00000	0.000
13	Shalmash	36º 05' N	45º 29' E	1622	Hap9(5), Hap10(1)	6	2	0.00032	0.333
14	Saqez	36º 03' N	46º 02' E	2168	Hap9(6)	6	1	0.00000	0.000
15	Benjun	36º 32' N	45º 31' E	2152	Hap11(6)	6	1	0.00000	0.000
	Total					77	1	0.00389	0.82399

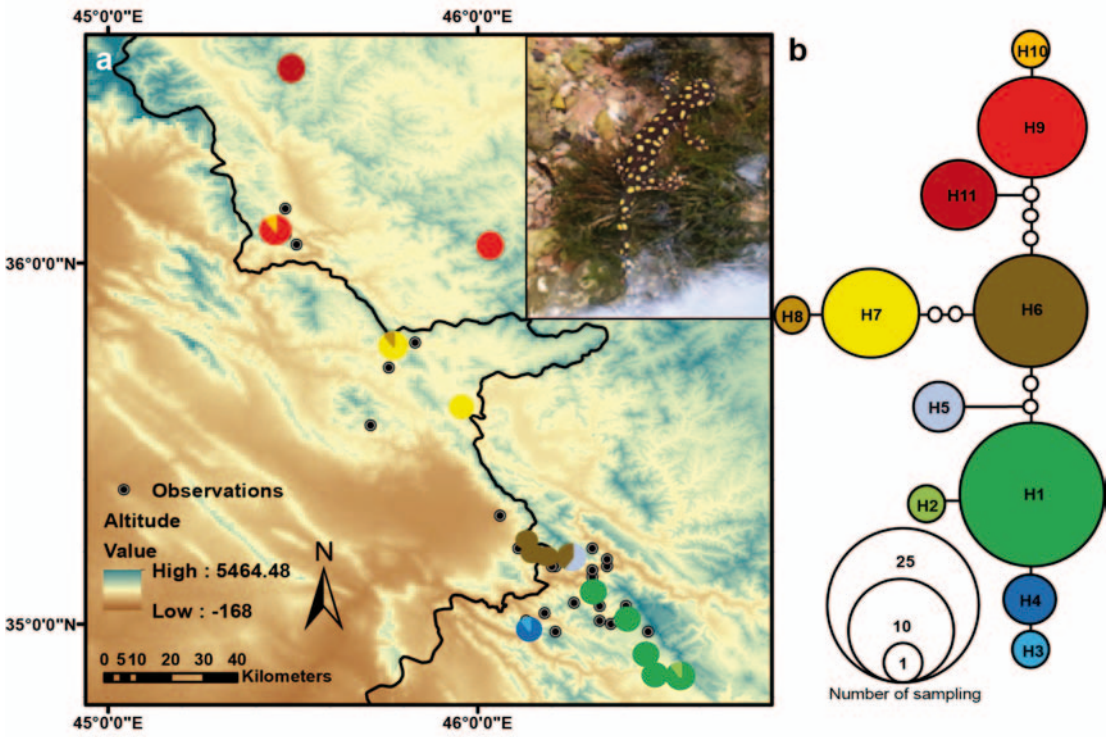

Figure 1. (a) Map illustrating the geographic distribution of *N. derjugini* and the 15 sampling localities in the study area (numbers show localities as indicated in Table 1) in Iran and Iraq; pies represent the haplotype frequency in each population that their colours are in accordance with haplotypes (H1-H11) in the haplotype network. **(b)** Haplotype network showing the phylogenetic relationships among the 11 haplotypes. Different haplotypes in the haplotype network have different colours. Sizes of circles are representative of the haplotype frequencies. Open dots represent missing intermediate haplotypes.

substitution models supported by our data. A consensus tree with posterior probabilities was generated using FigTree v1.4.0 (Rambaut, 2012). Furthermore, we applied a nested clade phylogeographic analysis (NCPA) using TCS v 1.21 for phylogeographic interpretation of relationships among haplotypes, (with a 95% parsimony connection limit, Clement et al., 2000).

Population analysis

Analysis of molecular variance (AMOVA) was conducted on (1) populations from three geographical regions as southern (populations 1-7), central (populations 9-12) and northern (populations 13-15), where selection of population groups was based on the haplotype groups designated in the phylogeny trees, and (2) all populations as one group to determine the level of genetic differentiation within and among *N. derjugini* populations, using Arlequin v 3.1 (Excoffier et al., 2005) with 10,000 permutations. Arlequin 3.1 (Excoffier et al., 2005) was also used to measure pairwise FST between populations.

Isolation by geographical and environmental distance

Mantel tests were used to evaluate the connection between geographical and environmental distances with genetic distances using Arlequin 3.1 (Excoffier et al., 2005). This analysis was performed based on a matrix of pairwise FST and a matrix of geographical distances as well as environmental distances with 10000 random permutations. We measured geographic distances between populations using DIVA-GIS v 7.5.0 (Hijmans et al., 2012). We used eight climate and land cover variables for our analysis that had previously been evaluated by Sharifi et al. (2017). These variables included precipitation of warmest quarter, precipitation of coldest quarter, temperature seasonality (standard deviation×100), isothermally (BIO2/BIO7×100), temperature annual range (BIO5–BIO6), mean temperature of driest quarter, mean temperature of wettest quarter and elevation. Environmental variables were processed in ArcMap 10.3 software and data matrices were analysed using SPSS version 16.0. In addition, a three-way Mantel test was performed between matrices of pairwise genetic distances and environmental distances, adding the matrix of geographical distances among populations.

Demographic analysis

Past population dynamics of *N. derjugini* was estimated with a Bayesian skyline plot (BSP) using BEAST, v 2.4.5 (Bouckaert et al., 2014). This analysis was carried out with the uncorrelated lognormal relaxed clock and the Bayesian skyline as a coalescent model with the mutation rate of 0.64% Myr (Weisrock et al., 2001). We ran the MCMC procedure with 100,000,000 generations, and the genealogy and parameters of the model were stored every 10,000 iterations. We used Tracer v 1.6 (Rambaut et al., 2014) to assess the effective population size through time.

Divergence time estimate

Four estimates of divergence between lineages of *N. derjugini* were obtained using BEAST v 2.4.5 (Bouckaert

et al., 2014). In all calibrations, we used a Bayesian Markov Chain Monte Carlo (MCMC) approach with the uncorrelated lognormal relaxed clock and the constant size as a coalescent model. The substitution model for each partition was obtained by Partition Finder v 2.1.1 (Lanfear et al., 2016). Runs were carried out based on 100 million generations, sampled every 1000 generations with the first 10% discarded as burn-in. We checked convergence and parameter estimates with ESS values >200 by Tracer v 1.6 (Rambaut et al., 2014). TreeAnnotator v1.8.4 (Drummond & Rambaut, 2007) was used to find the maximum credibility tree. In the absence of a fossil record of Neurergus and internal calibration points to calibrate the rate of divergence, we used external calibration points based on the estimated divergence time between *N. kaiseri* and *N. struchii* by Zhang et al. (2008). The root ages were 19.1 (12.1-26.4) Myr and 9.5 (5.4-13.8) Myr in calibration I and calibration II, respectively. Calibration III was carried out based on the evolutionary rate of the ND2 gene in salamanders identified by Weisrock et al. (2001) as 0.64% per Myr per lineage. Finally, calibration IV was carried out based on one fossil by approximating the crown of the genus *Triturus* dated at 24 Myr (Weisrock et al., 2001). In this analysis, in addition to the previous outgroups (*N. crocatus*, *N. kaiseri*, *N. strauchii*, *T. karelinii*, and *Ommatotriton vittatus*), we used two sequences of *T. carnifex* and one sequence each from *T. dobrogicus*, *T. cristatus*, *T. pygmaeus* and, *T. marmoratus* (available in GenBank under accession numbers: GQ258952, GQ258962, JN831597, NC_015790, GU982456 and, GQ258948).

RESULTS

We identified 11 unique haplotypes among 77 *N. derjugini* individuals based on the mitochondrial ND2 sequence (1036 base pairs), with 63 base pairs of the tRNA-Met gene at the beginning of the sequence. The ND2 mtDNA fragment contained a low polymorphism with only 16 variable sites, of which 14 were parsimony informative and 2 were singleton-variables including 14 transitions and 2 transversions (Table 2). Mean nucleotide compositions were A: 36.15 %, T: 25.36 %, C: 26.55 % and G: 11.94 %. The haplotype and nucleotide diversities across all populations of *N. derjugini* were 0.82399 and 0.00389, respectively. The haplotypes were allocated to different localities across the species' range. Seven of the eleven haplotypes were unique to their population, and two were shared only in two populations. Haplotype 1 was most widespread and abundant, shared among five of the fifteen populations. Ten populations had a single haplotype and the highest haplotype diversity (*Hd* = 0.60) occurred in Nowdeshe from the southern portion of the distribution range (Table 1). Average sequence divergence among *N. derjugini* haplotypes was low (0.54 ± 0.06%), whereas divergence of *N. derjugini* with *N. kaiseri* and *N. crocatus* was 6.8 ± 0.7% to 5.9 ± 0.7% respectively.

Bayesian and ML phylogenetic analyses based on 11 haplotype sequences of *N. derjugini* and five outgroup taxa had the identical topology (Fig. 2). All samples from 15 populations throughout the distribution range formed

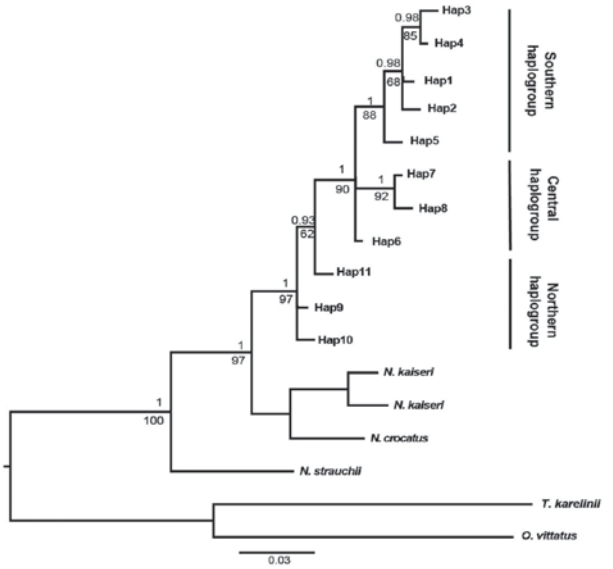

Figure 2. Phylogenetic trees of haplotypes implemented in PhyML and MrBayes based on partial ND2 gene sequence of 77 individuals for *N. derjugini* (Bayesian posterior probability values are above the branches, maximum likelihood bootstrap values are below the branches).

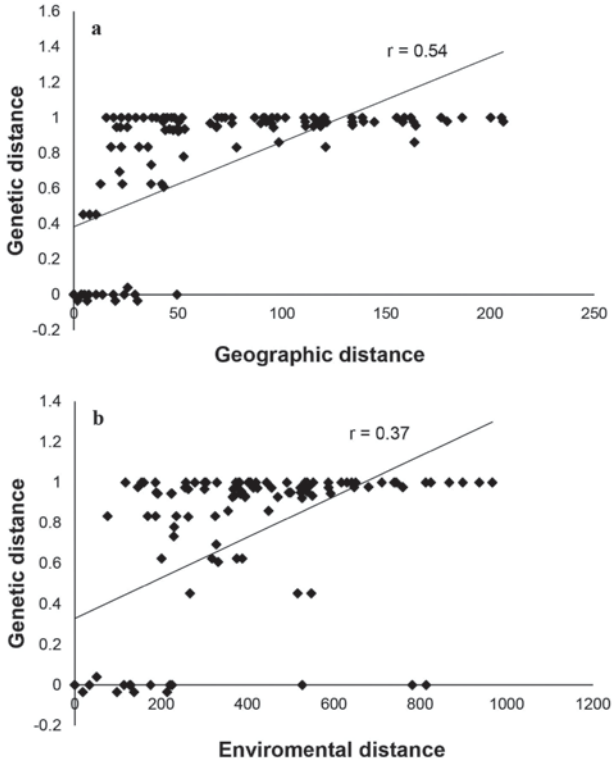

Figure 3. The plot of simple Mantel test indicating the correlation between **(a)** the geographic and genetic distances **(b)** environmental distance with genetic distance among 15 populations of *N. derjugini*.

a monophyletic group with high support (posterior probability = 1.00, likelihood bootstrap proportion = 97). The distribution of haplotypes was consistent with a northern, central, and southern part of the range. The TCS analysis (Fig. 1b) supported this phylogenetic tree. The geographic distribution of haplotypes suggests that gene

Table 2. Variable nucleotide sites and genetic variation within the partial sequences of the ND2 gene for 11 haplotypes of 77 Neurergus derjugini individuals in different localities

Hap	Polymorphic site																	Locality													Total
1	183	249	319	342	370	453	567	661	723	756	774	813	828	930	939	984	Ka	Ghor	Ghol	Do	Da	La	No	Ha	Ba	Ta	Pe	Si	Sh	Sa	Be	
2	A	G	A	A	G	A	A	T	T	C	G	C	A	C	C	C	5	5	5	5	5	0	0	0	0	0	0	0	0	0	0	25
3	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
4	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	T	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
5	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	4
6	.	.	G	G	.	.	.	.	.	.	.	.	.	.	.	.	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	3
7	C		.	G	.	.	.	C	.	.	.	.	.	.	.	.	0	0	0	0	0	0	2	4	4	4	0	0	0	0	0	14
8	C	.	.	G	.	.	.	C	.	.	A	T	G	.	.	.	0	0	0	0	0	0	0	0	0	0	4	6	0	0	0	10
9	C		.	G	.	.	.	C	.	T	A	T	G	.	.	.	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
10	C	.	.	G	.	C	G	C	C	.	.	.	.	.	T	.	0	0	0	0	0	0	0	0	0	0	0	0	5	6	0	11
11	C	.	.	G	.	C	G	C	C	T	.	.	.	.	T	.	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
	C	.	.	G	.	C	.	C	C	.	.	.	.	T	T	.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	6
Sample size																	6	5	5	5	0	5	4	4	4	4	5	6	6	6	6	77
Number of polymorphic sites																	1	0	0	0	0	1	3	0	0	0	1	0	1	1	0	16
Number of transitions																	1	0	0	0	0	1	2	0	0	0	1	0	1	0	0	14
Number of transversions																	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	2
Number of parsimony informative sites																	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	14
Number of nucleotide difference																	0.3	0	0	0	0	0.40	1.80	0	0	0	0.40	0	0.33	0	0	4.03
																	3					0	0				0		3			

Ka =Kavat, Ghor= Ghori ghale, Ghol= Gholani, Do= Dourisan, Da= Darrenajjar, La= Lashkargah, No= Nowdeshe, Ha= Hani garmale, Ta= Tawale, Ba= Balkha, Pe= Penjwin, Si= Siya gwez, Sh= Shalmash, Sa= Saqez, Be= Benjun.

Table 3. Results of analysis of molecular variance (AMOVA) using partial ND2 gene

Structure	Source of variation	d.f.	Variation (%)	F_{SC}	F_{ST}	F_{CT}
Three region	Among regions	2	74.91	0.84**	0.96**	0.74**
	Among populations within regions	12	21.13			
	Within populations	62	3.97			
The studies samples	Among populations		94.79			
	Within populations		5.20		0.94**	

Significant values are shown for $p < 0.05$ (*) and $p < 0.01$ (**)

flow between northern and southern regions is low.

The AMOVA revealed that most genetic variation was explained by differences among regions (74.91 % genetic variation: F_{CT} = 0.74, P < 0.01) followed by populations within regions (21.13 % genetic variation: F_{SC} = 0.84, P < 0.001; Table 3). Similar significant genetic differences exist among populations without the grouping (Table 3). Table 4 shows pairwise *F*st between populations.

There was a significant correlation between pairwise genetic distances and Euclidean distances (two-way Mantel tests, r = 0.54, P < 0.0001; Fig. 3a), a well as environmental distances (r = 0.37, P = 0.0012; Fig. 3b). Results of the three-way Mantel test revealed that the correlation between genetic and geographical distances remained significant even after accounting for the effect of environmental distance (r = 0.42, P = 0.0001). On the other hand, the elimination of the influence of geographical distance in the partial Mantel test resulted in a non-significant association between genetic and environmental distances (r = -0.097, P = 0.183).

The Bayesian skyline plot (Fig. 4) suggests that the population size was relatively stable from about 80,000 years ago (the middle Pleistocene) until approximately 25,000 years ago near the Last Glacial Maximum (LGM), followed by a contraction and an increase in effective population size starting at about 12,000 years ago near the Pleistocene-Holocene transition period.

Estimates of divergence between haplogroups in four calibrations are shown in Fig. 5. Calibrations I and II were the youngest and oldest, and estimation times by calibrations III and IV were between calibration I and II, while divergence times of calibration III was closer to

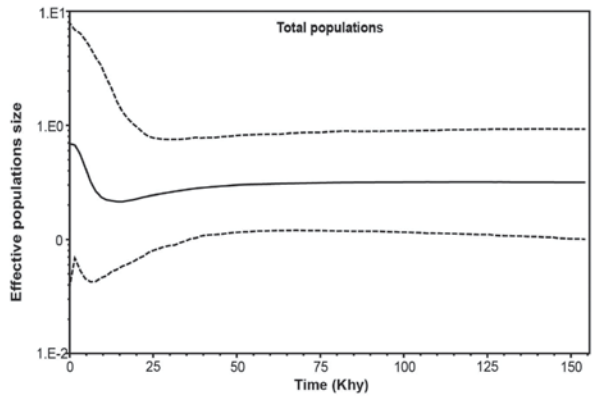

Figure 4. Bayesian skyline plot (BSP) based on partial ND2 sequences of *N. derjugini*. The x-axis shows time in the past in thousands of years, and the y-axis shows Ne (effective population size). Dashed lines show the median estimates, and white areas between the blue lines show the 95 % highest posterior density (HPD) limits.

calibration I. Taken together, divergence between the south-central and northern haplogroups took place in the early or middle Pleistocene (95% HPD, approximately 0.66 – 1.03 Myr), and divergence between southern and central haplogroups took place in the middle Pleistocene (95% HPD, approximately 0.41 – 0.83 Myr).

DISCUSSION

The present study revealed a low nucleotide diversity and a relatively high haplotype diversity for the total populations of *N. derjugini*. Haplotype diversities within ten populations of *N. derjugini* in our study were zero. Additionally, mean sequence divergence between all haplotypes (0.54 ± 0.06%) was low. Also, population genetic analyses exhibited significant phylogeographic structure in this species. There are reports of strong phylogeographic structure and low level of genetic divergence in several species of amphibians (Matsui et al., 2008, Richter et al., 2009, Farasat et al., 2016), which has been attributed to high frequency of inbreeding due to small population sizes, habitat loss, low dispersal ability (Allentoft & O'Brien, 2010), relatively short evolutionary history (Wang et al., 2017), a recent range expansion from glacial refugia (Makowsky et al., 2009; Pabijan et al., 2015; Vásquez et al., 2013), and a slow evolutionary rate at the genomic level (Chen et al., 2012). We expected genetic diversity of these populations to be low, due to a small geographical range and fragmentation of terrestrial habitat (Afroosheh et al., 2016), local extinctions (Sharifi & Assadian, 2004), and a small home range (Sharifi and Afroosheh, 2014).

Our phylogenetic analyses showed that all sampled populations form a monophyletic group. Average sequence divergence among *N. derjugini* and *N. kaiseri*, and between *N. derjugini* and *N. crocatus*, are 6.8% and 5.9% respectively. However, the average sequence divergence among populations of *N. derjugini* is only 0.4%. Although populations in different regions have specific haplotypes, there are very few mutational steps between the haplotypes. A similar study on *N. derjugini* conducted by Hendrix et al. (2014) based on mitochondrial genes (12S ribosomal RNA and control region) and one nuclear gene (KIAA gene) indicated that there are low genetic differences between populations separated as *N. microspilotus* and *N. derjugini*.

Mantel tests demonstrated that differentiation between populations of *N. derjugini* is more associated with geographic distances rather than environmental distances, a pattern that is typical for species with low dispersal capacity and low habitat availability (Dixo et al., 2009). Due to limited gene flow, geographically separated populations will become isolated even in the absence of barriers. Genetic drift and inbreeding will reduce genetic diversity in such populations (Irwin, 2002; Louy et al.,

Table 4. F_{ST} values between populations. Numbers are representative of localities based on Table 1.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	0.000														
2	-0.034	0.000													
3	-0.034	0.000	0.000												
4	-0.034	0.000	0.000	0.000											
5	-0.034	0.000	0.000	0.000	0.000										
6	0.733	0.833	0.833	0.833	0.833	0.000									
7	0.607	0.625	0.625	0.625	0.625	0.694	0.000								
8	0.934	1.000	1.000	1.000	1.000	0.945	0.452	0.000							
9	0.934	1.000	1.000	1.000	1.000	0.945	0.452	0.000	0.000						
10	0.934	1.000	1.000	1.000	1.000	0.945	0.452	0.000	0.000	0.000					
11	0.942	0.967	0.967	0.967	0.967	0.945	0.780	**0.928**	**0.928**	**0.928**	**0.000**				
12	0.972	1.000	1.000	1.000	1.000	0.975	0.830	**1.000**	**1.000**	**1.000**	**0.040**	**0.000**			
13	0.954	0.974	0.974	0.974	0.974	0.956	0.833	**0.950**	**0.950**	**0.950**	**0.950**	**0.976**	**0.000**		
14	0.976	1.000	1.000	1.000	1.000	0.978	0.860	**1.000**	**1.000**	**1.000**	**0.975**	**1.000**	**-.0.000**	**0.000**	
15	0.976	1.000	1.000	1.000	1.000	0.978	0.860	**1.000**	**1.000**	**1.000**	**0.975**	**1.000**	**0.923**	**1.000**	**0.000**

Node no.	Calibration I	Calibration II	Calibration III	Calibration IV
1	4.03 (2.69-5.23)	8.14 (5.94-10.51)	5.29 (4.08-6.65)	6.29 (4.93-8.33)
2	0.66 (0.35-1.04)	1.33 (0.72-2.11)	0.86 (0.29-0.83)	1.03 (0.57-1.63)
3	0.41 (0.21-0.66)	0.83 (0.44-1.35)	0.53 (0.29-0.83)	0.65 (0.34-1.03)
4	0.30 (0.11-0.53)	0.61 (0.23-1.07)	0.39 (0.16-1.29)	0.47 (0.17-0.82)
5	0.26 (0.06-0.54)	0.54 (0.13-1.09)	0.35 (0.09-0.68)	0.42 (0.11-0.85)
6	0.22 (0.09-0.39)	0.45 (0.18-0.80)	0.29 (0.12-0.49)	0.35 (0.14-0.62)
7	0.13 (0.004-0.27)	0.28 (0.08-0.54)	0.18 (0.06-0.33)	0.21 (0.06-0.41)
8	0.07 (0.003-0.17)	0.15 (0.009-0.36)	0.09 (0.006-0.22)	0.11 (0.006-0.28)
9	0.04 (0.001-0.13)	0.09 (0.002-0.26)	0.06 (0.002-0.17)	0.07 (0.002-0.20)
10	0.07 (0.002-0.20)	0.14 (0.003-0.40)	0.09 (0.004-0.35)	0.11 (0.004-0.31)
11	0.09 (0.002-0.27)	0.20 (0.005-0.55)	0.12 (0.004-0.35)	0.15 (0.004-0.42)

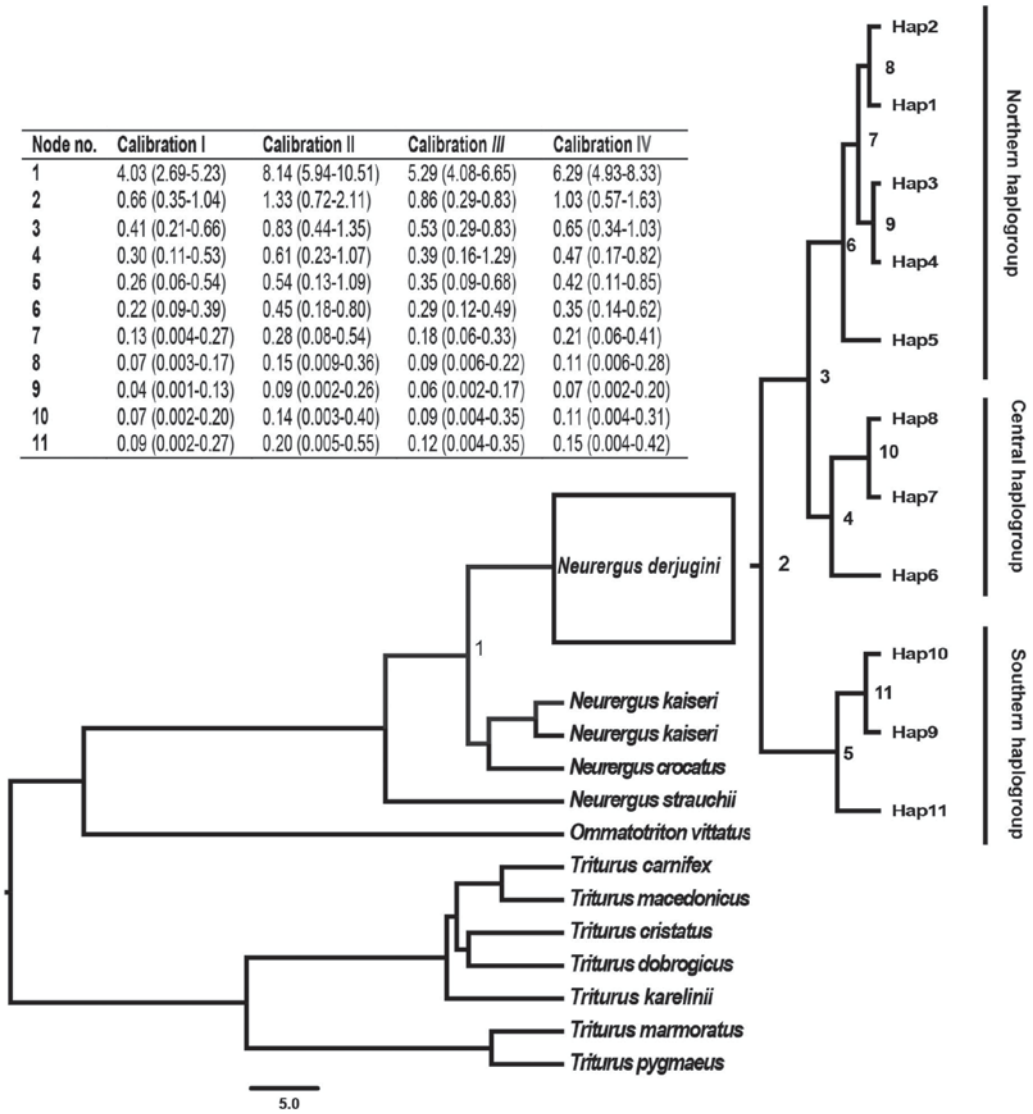

Figure 5. Chronogram of diversification implemented in BEAST based on partial ND2 gene for *N. derjugini*. The table shows three calibrations and the ranges of the divergence times for nodes in millions of years with 95 % highest posterior density (95 % HPD).

2007). The isolation by geographic distance mechanism has been reported in populations of *N. kaiseri*, the sister species of *N. derjugini* (Farasat et al., 2016). In the present study, the Mantel test revealed a positive but statistically non-significant correlation between genetic and environmental distances. Contrary to our expectations, distinct ecological parameters had a less strong influence on the genetic divergence among populations.

BSP analysis indicated an overall stationary historical population size with a contraction around the LGM followed by an expansion at the Pleistocene-Holocene transition. The classic scenario based on glacial contraction and postglacial expansion as is known in some species that are located in regions with higher latitude may not happen in mid-latitude areas such as Iran (Kehl et al., 2009). Different species in the Middle East may also respond in a different way. For example, Najafi et al. (2018) showed that divergence between two major geographical clades of *Rhinolophus euryale* (Chiroptera) in the Pleistocene was congruent with the classic scenario. However, Shahabi et al. (2017) reported contraction of populations in another Rhinolophid bat, *R. euryale*, in glacial periods within glacial refugia in southern Zagros Mts. It was also suggested by Ahmadzadeh et al. (2013) that there was a refuge in a narrow Zagros corridor between the Sabalan and Bozghosh mountain ranges during glacial periods for Iranolacerta brandtii (Reptilia). However, Javanbakht et al. (2017) reported that Transcaucasian tortoises had a long-term range stability and did not show shift in their range during glaciation and interglaciation.

The genetic variance observed within *N. derjugini* populations and its geographical distribution suggests that historical isolation has probably played a role in shaping the genetic structure of *N. derjugini*. Divergence dates based on four calibrations estimated that the most ancient diversification have probably occurred in haplogroups distributed in the south, centre and north during the early or middle Pleistocene, probably relating to the oscillating glacial cycles. Haplogroups of southern region diversified approximately around the LGM. Since the number of first order breeding streams and newt

abundance (as reported by the number of visual counts) are substantially higher in the southern region of the distribution (Afroosheh et al., 2016), it seems that *N. derjugini* expanded to surrounding areas and created extant distribution patterns with the combination of low nucleotide diversity and high haplotype diversity.

The Zagros open woodland of mostly oak in western Iran and eastern Iraq has experienced forest expansion and contraction as the result of fluctuating climate during the Pleistocene (Khalyani et al., 2013). Moreover, this area has been affected by livestock grazing and agricultural development since the beginning of the 5th Millennium BP (Wright et al., 1967). Long term traditional land use for grazing livestock by nomads and other disturbances associated with recent population growth are two main driving factors that have resulted in massive deforestation or changes in the vertical structure, composition, and configuration of forests in the Zagros Mountain Range (Metzger et al., 2005). The remnants of formerly widespread open woodlands are currently present only in the southern part of the geographic range of *N. derjugini*. The few remaining populations of *N. derjugini* in the northern part of its distribution are located in areas that presumably lost their natural vegetation cover decades ago (Afroosheh et al., 2016).

Low levels of genetic variation were observed among most populations of *N. derjugini*. Whether this low diversity is a threat to any of these populations has not been documented, and many of these populations may persist despite this. Nevertheless, a general correlation between population fitness and genetic diversity has been demonstrated in many groups of vertebrates including amphibians (Reed & Frankham, 2003; Jordan et al., 2009). The maximum linear distance between the most segregated breeding streams in the southern and northern parts of the species range is only 205 km. However, localities inhabited by *N. derjugini* are separated with nearest neighbour distances averaging 7.95 km. Surveys on the abundance of *N. derjugini* in 32 of the 42 localities within the Iranian range of the species resulted in the total visual count of 1,379 adults, juveniles, and larvae (mean/stream = 43; range, 1–601). Most of these observations (51%) were found in just two of the localities, 44% were found in 14 streams, and the remaining 5% were scattered among 16 streams (Afroosheh et al., 2016).

Very low levels of genetic variation within each small population and the lack of connectivity among most populations of *N. derjugini* occurring in fragmented habitats suggest that the species is at high risk of becoming extinct. Considering the isolation of many *N. derjugini* populations, it would seem reasonable to focus on management efforts to minimise future genetic drift and inbreeding by increasing population sizes and habitat connectivity. This is probably best accomplished by improving or expanding the available wetland habitats at each site to facilitate a natural population increase. We also recommend the supplementation of extant populations with captive bred individuals, a strategy which is enabled by the existence of a captive breeding facility for this species (Sharifi & Vaissi, 2014; Vaissi & Sharifi, 2018).

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