

Genetic Variation among various populations of spadefoot toads (*Pelobates syriacus*, Boettger, 1869) at breeding sites in northern Israel

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Received 1 August 2013; revised 9 September 2013; accepted 21 September 2013

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ABSTRACT

A genetic study was carried out on spadefoot toads (*Pelobates syriacus*) from habitats of various locations and altitudes in northern Israel. Cytochrome *b* and 12S were amplified by PCR for the analysis of genetic variation based on five DNA polymorphisms and for RAPD PCR. The nucleotide sequences of the mitochondrial DNA fragments were determined from a 460 bp clone of cytochrome *b* and a 380 bp clone of 12S (GenBank accession numbers, FJ595199-FJ595203). No genetic variation was found among the populations with regard to 12S. According to the analysis of five sequences using Arlequin software, there was a high gene identity among the populations (98.7% - 99.6%). Both populations, Elrom Pond breeding site, at the highest altitude and Fara Pond, at the lowest, had the lowest identities as compared to other populations. The DNA variation among *P. syriacus* populations from various breeding sites, according to band sharing (BS), when using the OP-4 primer, was 0.92 - 1.00. Similarity was low between the population of Elrom Pond and the populations of Kash Pond, Raihania Pond and Sasa Pond (0.92 BS), as well as between the population of Fara Pond at the lowest altitude, and the populations of Kash Pond, Raihania Pond and Sasa Pond (0.92 BS). Similar results were obtained when comparing the results obtained using primer OPA-3. The lowest similarity was found between populations of the highest altitude (Elrom Pond) and lowest altitude (Fara Pond), relative to the other ponds (Kash Pond, Raihania Pond and Sasa Pond) with a BS of 0.93.

Keywords: Spadefoot Toads; *Pelobates syriacus*; Cytochrome *b*; 12S; DNA Fragments

1. INTRODUCTION

Spadefoot toads (*Pelobates syriacus*) are distributed in: Azerbaijan (*syriacus*), Bulgaria (*balcanicus*), Greece (*balcanicus*), Iran (*syriacus*), Iraq (*syriacus*), Israel (*syriacus*), Lebanon (*syriacus*), Romania (*balcanicus*), Russia (*syriacus*), Syria (*syriacus*) and Turkey (*syriacus*). They belong to Pelobatidae, with only one genus, *Pelobates*, which contains four species [1-3]. Dzukic *et al.* [3] reported that the distribution range of *P. syriacus* in the Balkans is much larger than the previously known, rather compact but disjunctive. The range of *P. fuscus* penetrates deeply into the Balkans, but has a rather fragmented distribution. The systematic relationships among *Pelobates* species have recently been studied by Garcia-Paris *et al.* [4]. They showed that a *P. cultripes*-*P. varaldii* clade is sister to a *P. fuscus*-*P. syriacus* clade. Eiselt [5] even considered *P. syriacus* to be a monotypic species. Ugurtas *et al.* [6] suggested that *P. Syriaicus* comprises at least three taxa: *P. s. syriacus*, *P. s. balcanicus* and a third lineage that they collected in Serbia Eggerti *et al.* [7] studied the phylogeographic structure in *P. fuscus* from 16 localities throughout Europe using mitochondrial cytochrome *b* gene sequence analysis.

Different aspects of biology, ecology and genetics of *P. syriacus* in Israel, at the southern border of its distribution, have been described. The distribution and habitat selection of the ponds were related to water availability during spring and the beginning of summer [8], larval growth and complete metamorphosis [9,10], burrowing behavior [11], physiology adaptations to various environments [12,13], biology and life cycle [14-16] and genetic variation among populations [17,18]. Nevo [17] suggested that *Pelobates* is best explained by the environmental variability model based on selection for homozygosity, as an adaptive strategy, in the relatively

constant and narrow subterranean niche. This hypothesis is derived from experiments on proteins of the spadefoot toads, *P. syriacus* and *P. cultripes*, from Israel and Portugal encoded by 32 gene loci, that were analyzed electrophoretically.

In the present paper, we use molecular sequence data of mitochondrial genes to analyze the genetic variation of *P. syriacus* populations from various breeding sites in northern Israel.

2. MATERIALS AND METHODS

2.1. Specimens

In order to analyze *P. syriacus* populations from various areas in northern Israel, five *P. syriacus* specimens were collected from each population. The tissues were studied by mitochondrial sequence analysis. As previously described by Degani [10], sampling of specimens was carried out randomly from the entire area of the water body by hand net or collected from the area surrounding the

five breeding sites of *P. syriacus* in northern Israel (**Figure 1** and **Table 1**).

2.2. DNA Extraction, Amplification and Sequencing

Genomic DNA was extracted from ethanol-preserved tissue samples (clipped or whole tail of larvae) with the QIAamp DNA Mini Kit that employed proteinase K lysis on the tissue, as well as specific DNA binding to the QIAamp silica-gel membrane, through which contaminants pass. DNA samples were visualized after electrophoresis on a 0.8% agarose gel, stained with ethidium bromide. DNA concentration was measured using Nano Drop 100 (ThermoFisherScientific, Wilmington, DE, USA).

DNA of *Pelobates* cytochrome *b* and 12S was amplified by PCR and analyzed by RAPD PCR. Primers for DNA amplification of 12S genes were based on those of Veith *et al.* [2], and of cytochrome *b* were derived from sequences determined in our laboratory (**Table 2**).

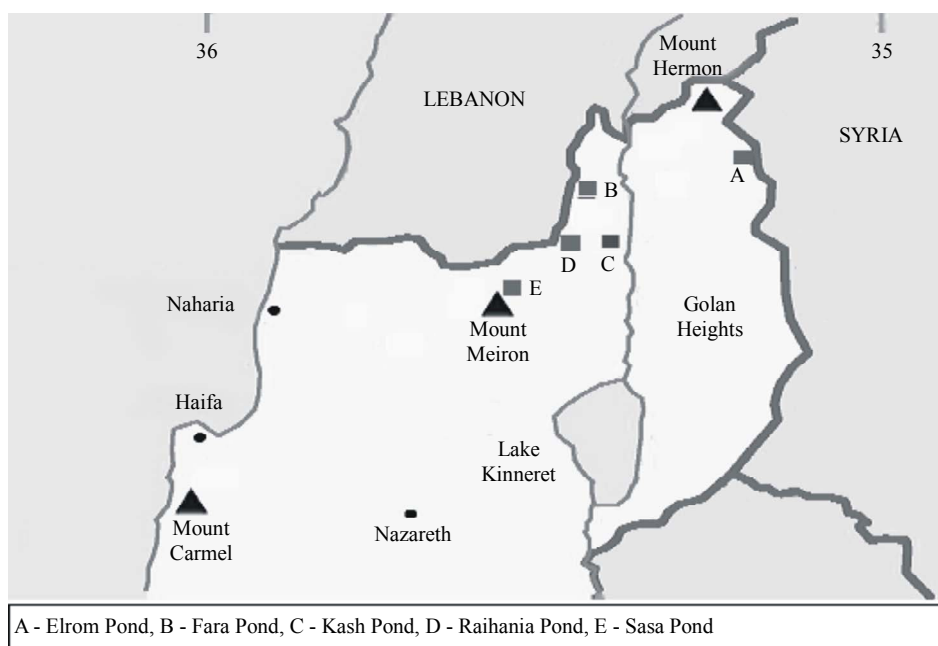


Figure 1. The breeding sites in northern Israel from which specimens of *P. syriacus* were sampled.

Table 1. Breeding sites in northern Israel from which specimens of *P. syriacus* were sampled.

Breeding sites	Latitude	Longitude	Altitude (m above sea level)
A—Elrom Pond	222677	286429	1060
B—Fara Pond	236119	157022	446
C—Kash Pond	270560	196203	809
D—Raihania Pond	272861	195791	665
E—Sasa Pond	270855	186972	810

Table 2. Primers used in this study.

PCR product length	Sequence 5'—3'	Primer	Species and genes
575 bp	GGAAACGTACTAGTCCAATG	Pcytfor1	Pelobates cytochrome b
	ATAAATGGTTCTTCAACAGG	Pcytrev1	
500 bp	AAA CTG GGA TTA GAT ACC CCA CTAT	12SA-L	Pelobates 12S
	GAG GGT GAC GGG CGG TGT GT	12SB-H	

PCR amplification was performed in a 50 µl solution containing 10 mM Tris-HCl, 50 mM KCl, 2.5 mM MgCl₂, 0.5 mM of each dNTP, 2 µM of primer, 10 - 500 ng genomic DNA and 2.5 units of Taq DNA polymerase (Promega, USA). The reaction was performed in a PTC-150 MiniCycler (MJ Research, USA) with the following parameters: 3 min denaturation at 94°C, followed by 36 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 52°C and elongation for 1 min at 72°C. An additional 5 min elongation period at 72°C followed the last cycle. After amplification, the PCR products were separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide. PCR products that had been purified using the HiYield Gel/PCR DNA Fragment Extraction Kit (RBC Bioscience, Taiwan) were sequenced at the Hy Laboratories (Rehovot, Israel).

2.3. DNA Sequence Analysis

The primer decamers (Mikulice and Pialek, 2003), OPA-3 (5'AGTCAGCCAC) and OPA-4 (5'AATCGGGCTG), were found to be best for sequence analysis of *Pelobates* by RAPD PCR. The reaction was performed in a 50 µl solution containing 10 mM Tris-HCl, 50 mM KCl, 2.5 mM MgCl₂, 0.5 mM of each dNTP, 2 µM of primer, 10 ng genomic DNA and 2.5 units of Taq DNA polymerase (Promega, USA), with the following parameters: 3 min denaturation at 94°C, followed by 40 cycles of 1 min at 94°C, annealing for 1 min at 35°C and elongation for 1 min at 72°C. An additional 5 min elongation period at 72°C followed the last cycle. After amplification, the PCR products were separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide.

The DNA variations study was executed in two steps. First, the variations in the populations were examined. Subsequently, the samples were run on one gel to find the genetic variations among the populations. To assess the similarity between individuals, band sharing (BS) of the RAPD PCR products was calculated as:

$$BS = 2'(Nab)/(Na + Nb),$$

where BS = level of band sharing between individuals *a* and *b*, Nab = number of bands shared by individuals, *a* and *b*, Na = total number of bands of individual *a* and Nb = total number of bands for individual *b* [19,20]. The

PCR patterns were compared only among samples that had been run on a single gel. Differences in BS were examined by the *d*-test for differences between proportions [21].

Multiple sequence alignments and phylogenetic cluster analysis were carried out using the MegAlign computer program (Windows32 MegAlign6.1, DNASTAR Inc.). Phylogenetic trees were generated by the Neighbour-joining method from distance matrices, which were based on multiple sequence alignments.

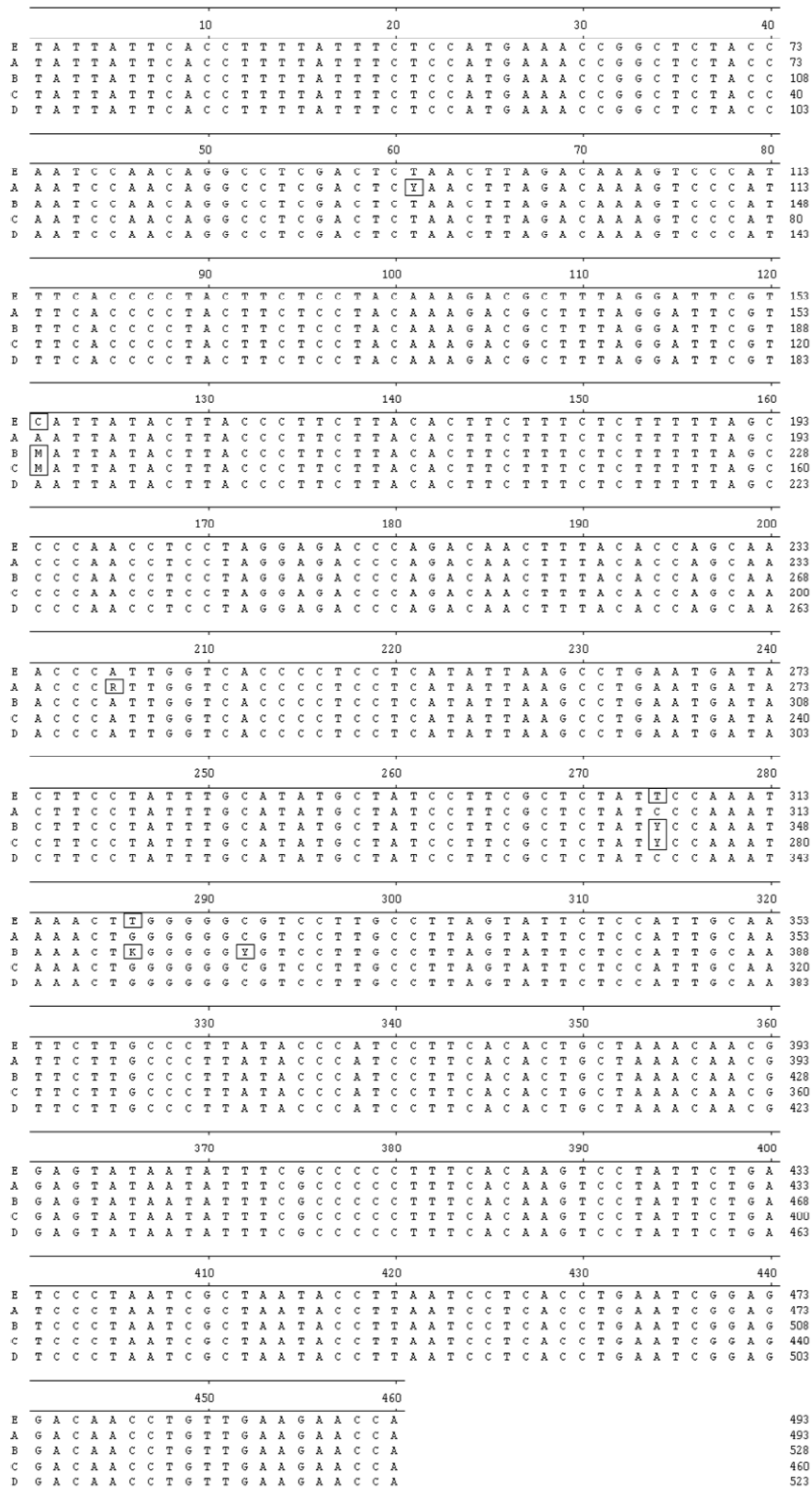
3. RESULTS AND DISCUSSION

The nucleotide sequences of the mitochondrial DNA fragments were determined from a 460 bp clone of cytochrome *b* (**Figure 2**) and a 380 bp clone of 12S. The cytochrome *b* fragment varied at the nucleotide sites, 61, 121, 205, 274 and 286 among populations of various breeding sites (**Table 3**) (GenBank accession numbers - FJ595199 to FJ595203). However, no genetic variation was found among the sequences of 12S (**Figure 3**).

A similar situation was revealed when comparing the cytochrome *b* gene variation among *P. syriacus* populations from various breeding sites (**Figure 4**). The analysis of five sequences by Arlequin software revealed a high gene identity (98.7% - 99.6%). In addition, it showed that Elrom Pond (A), the breeding site at the highest altitude, and Fara Pond (B) at the lowest altitude, had lower identities than other populations (**Figure 4**).

The DNA variation among *P. syriacus* populations of various breeding sites, according to the primers, OPA-3 and OPA-4, is presented in **Figure 5**. There were 7 - 8 identical bands when using OPA-3 and 11 - 12 identical bands when employing OPA-4. With primer OPA-4, the BS among populations varied between 0.92 - 1.00 (**Table 4**). The lowest similarity was revealed between the highest Elrom Pond (A) and population and those of the ponds, Kash Pond (C), Raihanian Pond (D) and Sasa Pond (E)(0.92 BS), as well as between the pond population of the lowest altitude, Fara Pond (B)(0.92 BS) (**Table 4**). Similar results were obtained in the analysis with the primer OPA-3. It seems that both altitude and the distance have effect on genetic diversities.

The genetic variation analysis of five populations in a small area in northern Israel revealed different results



Decoration 'Decoration #1': Box residues that differ from the Consensus.

A – Elrom Pond, B – Fara Pond, C – Kash Pond, D – Raihania Pond and E – Sasa Pond

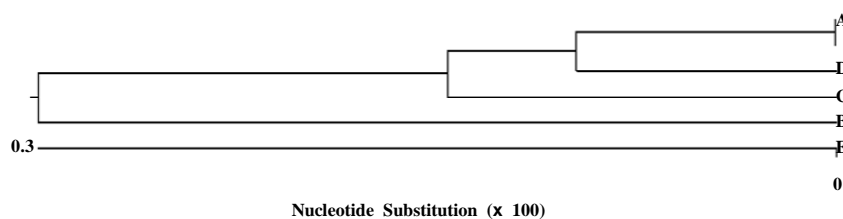
Figure 2. Alignment of the nucleotide sequences of the cytochrome *b* fragment of *P. syriacus* larvae from five breeding sites in northern Israel.

Table 3. The nucleotide sequence variations and GenBank accession numbers of cytochrome *b* fragments.

GenBank accession number	286	274	205	121	61	
FJ595199	T	T	A	C	T	A—Elrom Pond
FJ595200	G	C	R	A	Y	B—Fara Pond
FJ595201	K	Y	A	M	T	C—Kash Pond
FJ595202	G	Y	A	M	T	D—Raihanian Pond
FJ595203	G	C	A	A	T	E—Sasa Pond

>CAACCTTCTCCGCCAGGGGACTACGAGCGCTAGCTTAAAACCCAAAAGACTTGGCGGTGCCCTACACCCACCTAGAGGA
 GCCTGTTCTATAATCGATACCCCGCTTACCTCACCAATTTTGGCCAAAACCGCCTGTATACCCGCTGCCTCAACTTA
 CCCTTTGAAGGTTAAAAAGTAAGTATAATGGTCTTTCACCAAAACGTCAGGTCAAGGTGCAGCGAATAAATTGGATAGAA
 ATGGGCTACATTTCTACCTAGAAAATTACGGAAAATCACTATGAAACCTGATTAGAAGGCGGATTAGCAGTAAAAAG

Figure 3. The 12S ribosomal RNA partial sequence of *P. syriacus* in northern Israel.



D	C	B	A	E	
99.3	99.3	99.1	98.9	-	E
99.6	99.1	98.7	-	-	A
99.1	99.6	-	-	-	B
99.6	-	-	-	-	C
-	-	-	-	-	D

Raihanian Pond	Kash Pond	Fara Pond	El-Rom Pond	Sasa Pond	
99.3	99.3	99.1	98.9	-	Sasa Pond
99.6	99.1	98.7	-	-	El-Rom Pond
99.1	99.6	-	-	-	Fara Pond
99.6	-	-	-	-	Kash Pond
-	-	-	-	-	Raihanian Pond

Figure 4. Unrooted phylogenetic tree and the percent identity of the partial *cb* fragment based on nucleotide sequence of the *P. syriacus*. The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of the substitution events. The phylogenetic tree was constructed using the MegAlign program (DNASTAR) by the CLUSTALW method. The branch length represents the evolutionary distance. A—Elrom Pond, B—Fara Pond, C—Kash Pond, D—Raihanian Pond and E—Sasa Pond.

depending on the genetic marker, molecular mitochondrial DNA (cytochrome *b* and 12S) and nuclear DNA (RAPD PCR). Cytochrome *b* was found to be less conservative than 12S, and it showed a low genetic variation when using RAPD PCR. This pattern appeared to be

mainly attributed to the sites of the *P. syriacus* populations. According to this analysis, a very high similarity (98.7% - 99.6) was found among the populations. Munwes *et al.*, 2010 [22] used the eastern spadefoot toad in Israel as a model system to examine these hypotheses

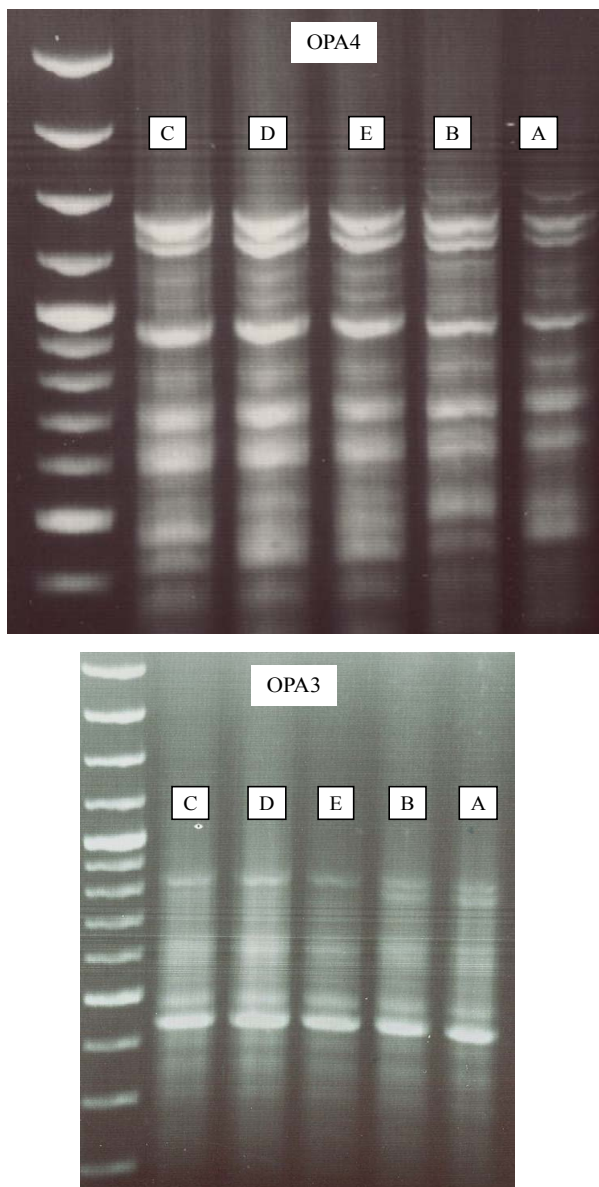


Figure 5. RAPD PCR results using the OPA-3 and OPA-4 primers. A—Elrom Pond, B—Fara Pond, C—Kash Pond, D—Raihanian Pond and E—Sasa Pond.

using population genetics analyses and network theory using mitochondrial DNA (only D-loop) region individuals sampled at different locations in Israel but not from various habitats and different altitudes in a small area as was done in the present study. They identified two distinct communities, which are genetically linked through two specific rain pools in the Upper Galilee. Details on the spatial subdivision of this species are cardinal for future management and restoration of temporary wetlands in Israel [22]. In the present study using different molecular markers (cytochrome *b* and 12S and RAPD PCR), the lowest similarity was found between populations of the highest altitude (Elrom Pond) and lowest

Table 4. The common bands and band sharing (BS) found in RAPD PCR with OPA-3 and OPA-4 primers between paired populations.

		OPA-4				
		A	B	E	D	C
	0.92	0.92		1.00	1.00	C
	0.92	0.92		1.00		D
	0.92	0.92				E
	1.00					B
						A
		OPA-3				
		A	B	E	D	C
	0.93	0.93		1.00	1.00	C
	0.93	0.93		1.00		D
	0.93	0.93				E
	1.00					B
						A

A—Elrom Pond, B—Fara Pond, C—Kash Pond, D—Raihanian Pond, E—Sasa Pond.

altitude (Fara Pond), relative to the other ponds (Kash Pond, Raihanian Pond and Sasa Pond) with a BS of 0.93.

Based on allozymic variations in proteins encoded by 32 gene loci, Nevo [17] suggested that the selection for homozygosity, as an adaptive strategy, seems to operate in the relatively constant and narrow subterranean niche. As compared to many studies on the phylogenetic, geographical and genetic relationships of Pelobatoidea [2,4,6,7], the information on genetic habitat selection in a relatively small area in Israel and the effect of ecological and environmental factors which might be influenced by altitude and other parameters, are very rare in this systematic group [17,18]. Many aspects of *P. syriacus* biology have been studied. It seems that all of them might be affected by environment. Adaptation to small differences among habitats is very difficult, but is possible. This has been demonstrated in other amphibians in the same area, e.g., in *Salamandra infraimmaculata*, according to RAPD PCR and the mt DNA method [23,24] and in *Triturus v. vittatus*, when using the RAPD PCR method [25]. Both *S. infraimmaculata* (see review [26]) and *T. v. Vittatus* [27,28] are found at different altitudes and breed in various water bodies, some of which dry up within a few months and others, which remain all year round. The biology and ecology of *P. syriacus* seem to have been studied less [14-16]. However, based on the results that the *P. syriacus* larvae can grow and complete metamorphosis only in relatively predictable breeding sites,

where water is available during most of the year [9,10] and taking into account the burrowing behavior of this species [11], which prevents dehydration, it seems that *P. syriacus* is less exposed to extreme conditions in northern Israel than *Triturus v. vittatus* and *S. infraimmaculata*. This might explain the low molecular variations among the various populations, as discovered in the present study and as apparently supported by Nevo [17] and Nevo and Beiles [18].

The above circumstances may explain the decline in *P. syriacus* [7], as shown by the relatively few breeding sites used by this species in northern Israel [8].

In conflation, this study shows that the environment (in different altitudes) and distains in the habitats of *P. syriacus* have an effect on the genetic molecular level.

REFERENCES

- [1] Roelants, K. and Bossuyt, F. (2005) Archaeobatrachian paraphyly and pangaean diversification of crown-group frogs. *Systematic Biology*, **54**, 111-126. <http://dx.doi.org/10.1080/10635150590905894>
- [2] Veith, M., Fromhage, L., Kosuch, J. and Vences, M. (2006) Historical biogeography of Western Palearctic pelobatid and pelodytid frogs: A molecular phylogenetic perspective. *Contributions to Zoology*, **75**, 109-120.
- [3] Dzukic, G., Beskov, V., Sidorovska, V., Cogalniceanu, D. and Kalezic, L.M. (2005) Historical and contemporary ranges of the spadefoot toads *Pelobates* spp. (Amphibia: Anura) in the Balkan Peninsula. *Acta Zoologica Cra-coviensis*, **48A**, 1-9.
- [4] Garcia-Paris, M., Buchholz, D.R. and Parra-Olea, G. (2003) Phylogenetic relationships of Pelobatoidea re-examined using mtDNA. *Molecular Phylogenetics and Evolution*, **28**, 12-23. [http://dx.doi.org/10.1016/S1055-7903\(03\)00036-8](http://dx.doi.org/10.1016/S1055-7903(03)00036-8)
- [5] Eiselt, J. (1988) Krottenfrosche (Pelobates gen., Amphibia, Salientia) in Turkisch-Thrakien und Griechenland. *Annalen des Naturhistorischen Museums in. Win. Serie B. fur Botanid und Zoologia*, **90B**, 51-59.
- [6] Ugurtas, I.H., Ljubisavljevic, K., Sidorovska, V., Kalezic, M.L. and Dzukic, G. (2002) Morphological differentiation of eastern spadefoot toad (*Pelobates syriacus*) populations. *Israel Journal of Zoology*, **48**, 13-32. <http://dx.doi.org/10.1560/UMHA-6J3N-FUG9-D7EV>
- [7] Eggert, C., Cogalniceanu, D., Veith, M., Dzukic, G. and Taberlet, P. (2006) The declining spadefoot toad, *Pelobates fuscus* (Pelobatidae): Paleo and recent environmental changes as a major influence on current population structure and status. *Conservation Genetics*, **2**, 185-195. <http://dx.doi.org/10.1007/s10592-006-9124-y>
- [8] Degani, G. and Kaplan, D. (1999) Distribution of amphibian larvae in Israeli habitats with changeable water availability. *Hydrobiologia*, **405**, 49-56. <http://dx.doi.org/10.1023/A:1003796820900>
- [9] Degani, G. (1982) Amphibian tadpole interaction in winter pond. *Hydrobiologia*, **96**, 3-7. <http://dx.doi.org/10.1007/BF00006274>
- [10] Degani, G. (1986) Growth and behavior of six species of amphibian larvae in winter pond in Israel. *Hydrobiologia*, **140**, 5-10. <http://dx.doi.org/10.1007/BF00006723>
- [11] Degani, G. and Carmali, D. (1988) Burrowing behavior of *Pelobates syriacus*. *Biology of Behaviour*, **13**, 22-29.
- [12] Degani, G., Goldenberg, S. and Warburg, M.R. (1983) Changes in ion, urea concentrations and blood plasma osmolarity of *Pelobates syriacus* juveniles under varying conditions. *Comparative Biochemistry and Physiology*, **75A**, 619-623.
- [13] Warburg, M.R. (1995) Hormonal effect on osmotic, electrolyte and nitrogen balance in terrestrial amphibian. *Zoological Science*, **12**, 1-11.
- [14] Degani, G. and Mendelssohn, H. (1990) Plants and animals of the land of Israel. 2nd Edition, The Publication House Society for Protection of Nature, Israel, 190-221.
- [15] Gafny, S. (1986) The biology and ecology of the Syrian spadefoot toad *Pelobates syriacus* in Israel. Tel-Aviv University, Tel-Aviv.
- [16] Gafny, S. (2004) Threatened amphibians of Israel. In: Dolev, A. and Perevolotsky, A., Eds., *The Red Book of Vertebrates in Israel. Israel Nature and National Parks Protection Authority and Society for Protection of Nature*, Israel Press, Jerusalem.
- [17] Nevo, E. (1976) Adaptive strategies of genetic systems in constant and varying environments. In: Karlin, S. and Nevo, E., Eds., *Population Genetics and Ecology*, Academic Press, New York, 141-158.
- [18] Nevo, E. and Beiles, A. (1991) Genetic diversity and ecological heterogeneity in amphibian evolution. *Copeia*, **1991**, 565-592.
- [19] Jeffreys, A.J. and Morton, D.B. (1987) DNA fingerprints of dogs and cats. *Animal Genetics*, **18**, 1-15
- [20] Wetton, J.H., Carter, R.E., Parkin, D.T. and Walters, D. (1987) Demographic study of a wild house sparrow population by DNA fingerprinting. *Nature*, **327**, 147-149.
- [21] Parker, R. (1976) Introduction of statistics for biology. Camelot Press, Southampton.
- [22] Munwes, I., Geffen, E., Roll, U., Friedmann, A., Daya, A., Tikochinski, Y. and Gafny, S. (2010) The change in genetic diversity down the core-edge gradient in the eastern spadefoot toad (*Pelobates syriacus*). *Molecular Ecology*, **19**, 2675-2689.
- [23] Degani, G., Jackson, K., Dosoretz, C. and Plotzky, Y. (1999) Molecular DNA variation in *Salamandra s. infraimmaculata* from different habitats. *Israel Journal of Zoology*, **44**, 239-246.
- [24] Goldberg, T., Pearlson, O., Nevo, E. and Degani, G. (2007) Mitochondrial DNA analysis of *Salamandra infraimmaculata* larvae from habitats in northern Israel. *Progrese și Perspective in Medicina Veterinară-Lucrări științifice*, **50**, 23-31.
- [25] Pearlson, O. and Degani, G. (2007) Molecular DNA variations among *Triturus vittatus* vittatus (Urodela) from different breeding sites at the southern limit of its distribution. *Acta Herpetological Journal*, **2**, 69-77.

- [26] Degani, G. (1996) The salamander at the southern limit of its distribution. Laser Pages Publishing, Israel.
- [27] Degani, G. and Mendelssohn, H. (1983) The habitats, distribution and life history of *Triturus vittatus vittatus* (Jenyns) in the Mount Meron Area (Upper Galilee, Israel). *British Journal of Herpetology*, **6**, 317-319.
- [28] Pearlson, O. and Degani, G. (2008) The life history of *Triturus v. vittatus* (Urodela) in various habitats. *Asiatic Herpetological Research*, **11**, 91-95.