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Genetic Variation in Xeric Habitats of *Triturus vittatus vittatus* (Urodela) Using Mitochondrial DNA of *12S* and*16S*, and Nuclear Gene, Rhodopsin, on the Southern Border of its Distribution

Gad Degani [1,2]

[1]MIGAL – Galilee Research Institute, KiryatShmona, Israel
[2]Faculty of Science and Technology, Tel-Hai Academic College, KiryatShmona, Israel

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Abstract: The present study examined the genetic variations of the newt (*Triturus vittatus vittatus*) (Urodela) from different breeding sites on thesouthern border of its distribution using mitochondrial DNA of *12S* and *16S*, and nuclear gene, rhodopsin. In an analysis of DNA sequences of rhodopsin (163 bp) from various breeding sites in different habitats, no genetic variation among the different populations was found. Moreover, using molecular variance (AMOVA) analysis no significant differences were detected. On the other hand, a great variance was found in comparing the two mitochondrial genes, the 12S–rRNA (357 bp) fragment and the 16S–rRNA (521 bp) fragment in regions of the different populations. A comparison between different populations using AMOVA showed great dissimilarity regarding the breeding sites of newts on thesouthern border of their distribution and at the highest altitudes.

Keywords: Genetic variations, Mitochondrial DNA, *Triturus*

Introduction

The banded newt *Triturus vittatus* (Litvinchuk *et al.*, 2005) is distributed throughout western Caucasus, Turkey, Lebanon, Syria, Israel and Iraq. The banded newt consists of two species, *Triturus ophryticus* and *Triturus vittatus*, based on trunk vertebrae count, genome size and allozyme data. The northern taxon, *Triturus ophryticus*, is subdivided into two geographic fragments: "western group" populations from western Anatolian Turkey; and "eastern group" populations distributed in the remaining area of Pontic Turkey and Western Caucasus. According to the above criteria, the *Triturus vittatus* species is found in Israel (Litvinchuk *et al.*, 2005). The biology of *Triturus vittatus* in the Mediterranean area has been described by Degani (2017) and Olgun *et al.* (1997). They have reported that there are two known subspecies in the genus *Triturus*: *T. v. vittatus* along the eastern edge

of the Mediterranean Sea from Turkey to Israel, where it reaches its southern limit; and *T. v. ophryticus* in the Caucasus, east and south of the Black Sea (Litvinchuk *et al.*, 2005). The banded newt, *T. v. vittatus*, is an endangered species in Israel (Pearlson and Degani 2007 a, b, 2008, 2011; Pearlson *et al*., 2010; Pearlson, 2012) at the southern limit of its distribution. The adaptation of *T. v. vittatus* on the southern border of newt populations in Israel has been scarcely described (Degani, 2017). Different genetic markers have been used in order to study the variation among different populations in the distribution of *T. v. vittatus* at the southern limit. It is difficult to explain the genetic variation among populations in a relatively small area, therefore the use of additional molecular markers is important. A previous study used different markers in studying the genetic variation of *T. v. vittatus*, including the random amplification of polymorphic DNA (RAPD) method (Pearlson and Degani, 2007a), and nucleotide sequences of the mitochondrial cytochrome b and the control region (Dloop) (Pearlson *et al*., 2010). The objective of the present paper is to study the genetic divergence among populations using the mitochondrial DNA of *12S* and *16S*, and the nuclear gene, rhodopsin.

Materials and Methods

Sites studied and larvae collected

Larvae of newts (*Triturus vittatus vittatus*) were collected randomly by a hand-net (Goldberg *et al.*, 2010) from winter ponds where newts were available. Their locations and descriptions are presented in Figure 1 and Table 1, respectively (Pearlson *et al*., 2010). Tissue samples were obtained from larvae (whole tails) and adults (clipped pieces of tails). Of these, two were breeding sites outside Israel, i.e. Syria (Damascus) and Turkey (European part), from which two tail-clipped tissue samples of *T. v. vittatus* and four tail-clipped tissue samples of *T. v. ophryticus* were collected, respectively. Samples, each consisting of 5-10 larval or adult *T. v. vittatus*, were collected from breeding sites (ponds) in Israel.

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, which uses proteinase K lysis of the tissue and 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 that was stained with ethidium bromide. The DNA concentration was measured using NanoDrop 100 (ThermoFisher Scientific, Wilmington, DE, USA)(Degani *et al*., 2013 b). DNA of *Triturus vittatus 12S* and *16S* was amplified by PCR and analyzed by RAPD PCR. Primers for DNA amplification of *12S* genes were based on those of Degani *et al*. (2013 b). PCR amplification was performed in a 50 µl solution containing 10 mM Tris-HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 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 1 min at 94 C, annealing for 1 min at 52 C and elongation at 72 C for 1 min. 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 that was stained with ethidium

bromide. PCR products were purified using HiYield Gel/PCR DNA Fragment Extraction Kit (RBC Bioscience, Taiwan) and sequenced at the Hy-Laboratories (Rehovot, Israel).

For the primer decamers (Mikulicek and Pialek, 2003), the reaction was performed in a 50 µl solution containing 10 mMTris-HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 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 at 72 C for 1 min. 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, which was stained with ethidium bromide.

Multiple sequence alignments and a phylogenetic cluster analysis were carried out using the MegAlign computer program (Windows32 MegAlign6.1, DNASTAR, Inc.)(Pearlson and Degani, 2007 a). Phylogenetic trees were generated by the neighbor-joining method from distance matrices that was based on the multiple sequence alignment (Pearlson *et al*., 2010).

Statistical analysis

The genetic similarity among samples was inferred using the neighbor-joining method (Saitou and Nei, 1987). Genetic distances were computed using the maximum composite likelihood method (Tamura *et al*., 2007) and are presented as the number of base substitutions per site. In order to examine the distribution of variations and differential connectivity among populations (the proportion of the total variance among populations (PhiPT), regions (the proportion of the total variance among regions (PhiRT), and populations within regions (the proportion of the total variance among populations within regions (PhiPR) (Table 4), we performed an analysis of molecular variance (AMOVA) with populations nested within regions using GenAlEXb (Peakall and Smouse, 2006).

Results

DNA sequences analyzed from rhodopsin (163 bp) are presented in Figure 1. There are no genetic variations among the different populations in comparison to the rhodopsin sequence. No differences are found between populations and within populations using AMOVA. The analysis of genes rhodopsin sequences among various breeding sites by AMOVA is 0 (Table 4). On the other hand, differences are found among populations in comparing the two genes, 12S and 16S (Figs. 3, 4).

Nucleotide differences in the mitochondrial gene 12S–rRNA (357 bp) fragment among *T. v. vittatus* samples from different breeding sites in Israel are presented in Figure 3. There are cases in which the sequences of newt mitochondrial DNA from different populations had high similarities in Israel. Four populations are shown to be highly dissimilar compared to populations in other breeding sites in the Jaudha Pond and Hermon (in Israel), and the Turkey and Damascus populations (Fig. 3).

In a comparison made by the AMOVA analysis, differences are found between and among the populations in the two genes, 12S and 16S (Table 4). The differences are significant between and among the populations. In comparing the 16S–rRNA (521 bp) fragment, three populations – Afeka, Berekhya, Turkey and Damascus – show a high variation compared to the others (Fig. 4).

Table 1: Breeding sites of *T. v. vittatus* on the southern border of its distribution

Breeding Site	**Latitude (N)**	**Longitude (E)**	**Altitude (meter ASL)**
Dovev Pond (1)	33°03'01''N	35°24'54''E	740
Nahalit Pond (2)	33°04'56''N	35°27'48''E	665
Matityahu Pond (3)	33°04'06''N	35°27'14''E	670
Pharaa Pond (4)	33°03'58''N	35°27'39''E	682
Amiad Water Holes (5)	32°55'03''N	35°33'04''E	212
Jaudha Pond (6)	32°56'42''N	35°36'49''E	110
Leshem Pond (7)	32°51'20''N	35°16'13''E	300
Afeka Pond (8)	32°06'52''N	34°48'42''E	15
Berekhya Pond (9)	31°39'32''N	34°38'44''E	20
Hermon(10)	33°01'48''N	35°29'26''E	815
Damascus, Syria	33°37'18''N	36°05'31''E	1150

Table 2: Samples of newts (*T. v. vittatus*) taken from different breeding sites for DNA analysis

Breeding Site	**Area**	**12S-rDNA**	**16S-rDNA**
Nahalit Pond (2)	Upper Galilee	7	8
Matityahu Pond (3)	Upper Galilee	13	14
Dovev Pond (1)	Upper Galilee	9	9
Pharaa Pond (4)	Upper Galilee	5	4
Amiad Water Holes (5)	Hula Valley	3	3
Jaudha Pond (6)	Hula Valley	1	1
Leshem Pond (7)	Lower Galilee	5	5
Afeka Pond (8)	Central Coastal	6	6
Berekhya Pond (9)	Southern Israel	3	3
Hermon(10)	Northern Israel	3	2
Damascus		2	2
Turkey		3	3

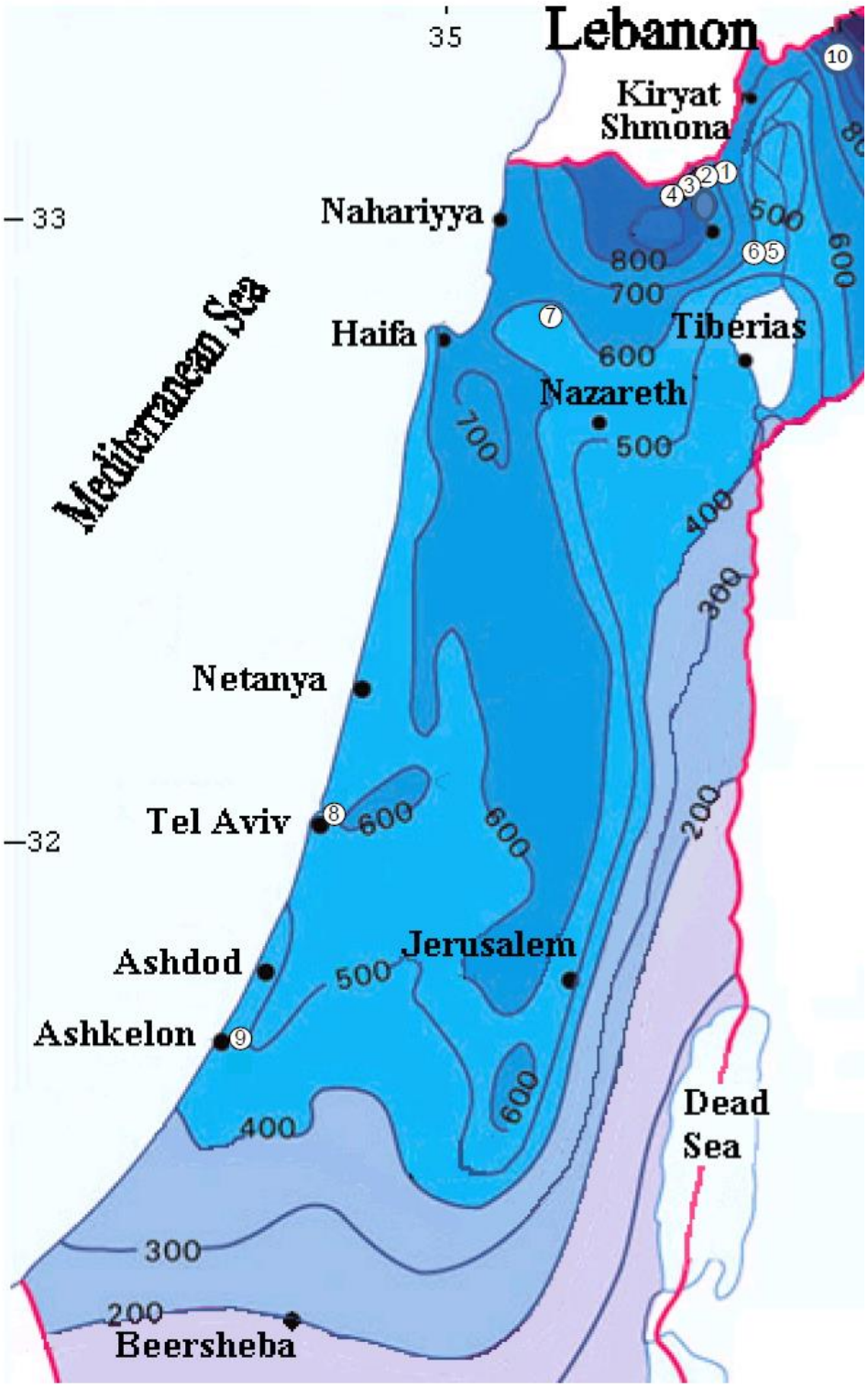


Figure 1: Map of newt sampling breeding sites of *T. v. vittatus* in Israel with average annual precipitation (mm) (Fein *et al.*, 2007). Numbers (in circle) correspond to the identification numbers in Table 1

Table 3: Primers used to amplify the rhodopsin

Analysis	Primer	Sequence 5-3'
Rhodopsin	trhodf	TGTCCCCTTCTCCAACAAGA
Rhodopsin	trhodr	GAACCCTCCAAAGACCATGA

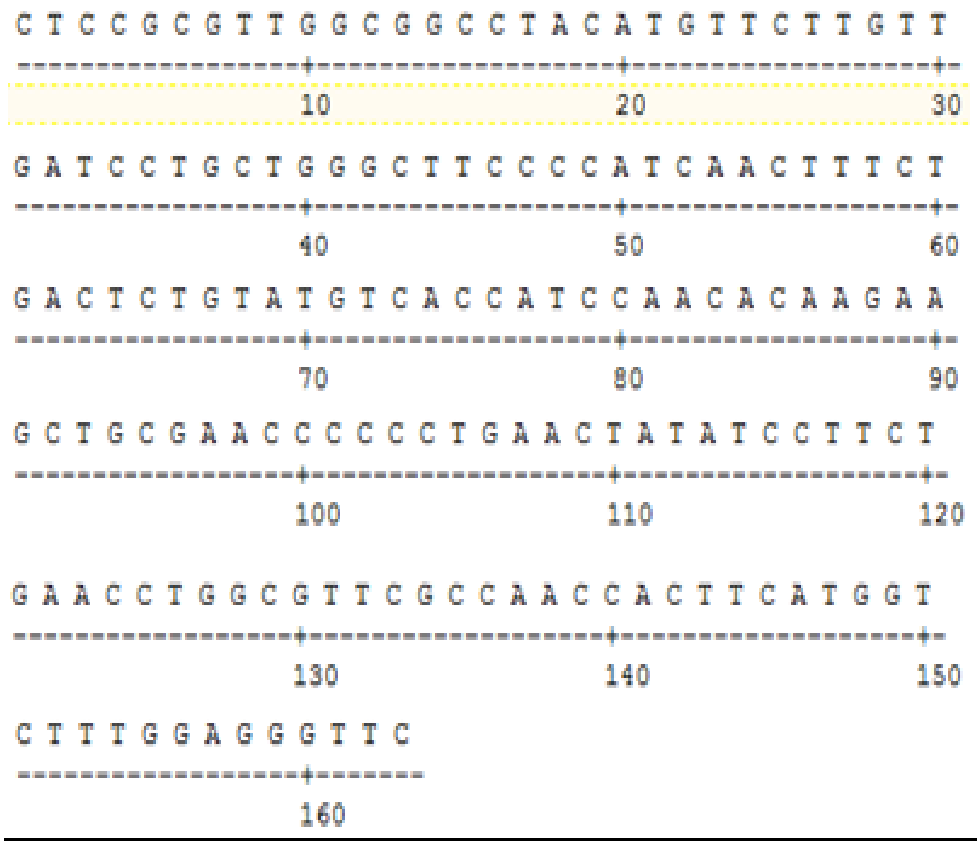
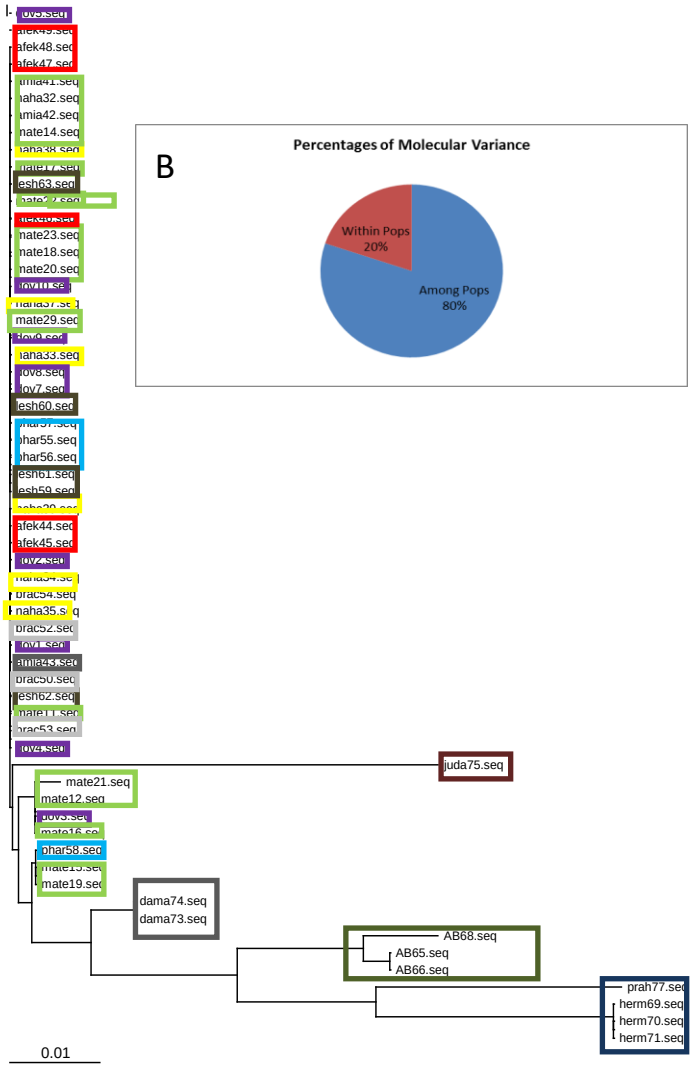


Figure 2: The rhodopsin sequence (163 bp)

Table 4: Analysis of gene sequences among various breeding sites by AMOVA

Gene	PhiPT value	P(rand >= data)	Nm
Rhodopsin	0	0.01	
12S-rDNA	0.802	0.01	0.124
16S-rDNA	0.636	0.01	0.286

A



B

Breeding Site
Nahalit Pond (2) –naha
Matityahu Pond (3)–mate
Dovev Pond (1) –dov
Pharaa Pond (4) –phar
Amiad Water Holes (5) –amia
Jaudha Pond (6) - juda
Berekhya (9) –brach
Leshem Pond (7) –lesh
Afeka Pond (8) –afek
Hermon (10)–herm
Damascus –dama
Turkey – AB65

Figure 3: A: Calculated standard parameters of nucleotide variation in the mitochondrial (mt) gene of the newts (*T. v. vittatus*) 12S–rRNA (357 bp) fragment represented in the phylogenetic tree among populations. The evolutionary history was inferred using the neighbor-joining method (Scale 0.01). B: AMOVA analysis.

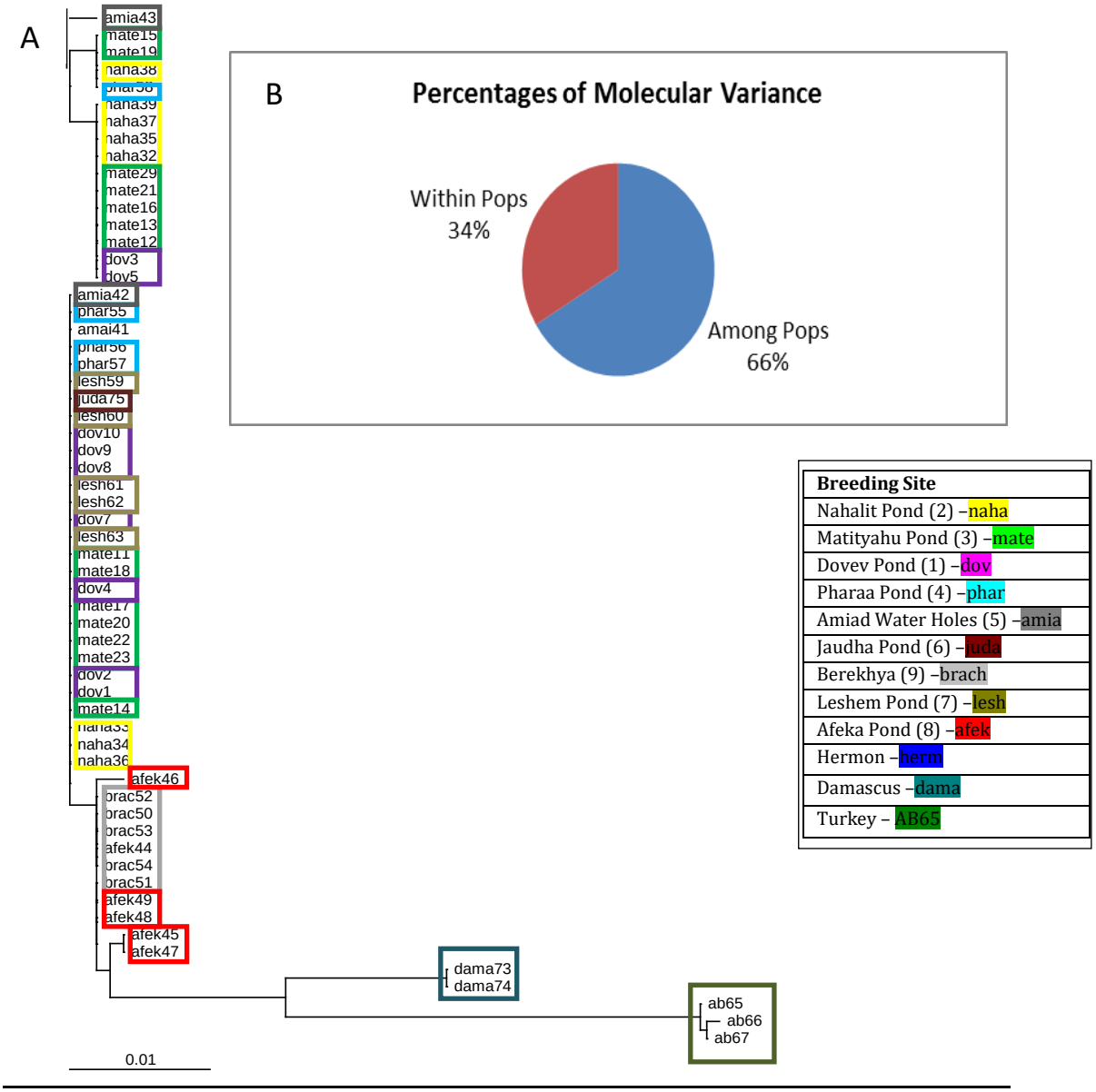


Figure 4: A: Calculated standard parameters of nucleotide variation in the mitochondrial (mt) gene of the newts (*T. v. vittatus*) 16S–rRNA (521 bp) fragment represented the phylogenetic tree among populations. The evolutionary history was inferred using the neighbor-joining method. (Scale 0.01). B: AMOVA analysis

Discussion

Many aspects of the genetic, biological and ecological divergence among various populations of the *Triturus vittatus* at the southern border of its distribution have been studied intensively (Warburg, 1971; Degani and Mendelssohn, 1983; Geffen *et al*., 1987; Staniszewski, 1995; Kutrup *et al*., 2005; Pearlson and Degani, 2007 a, b, 2008, 2011; Pearlson *et al*., 2010; Pearlson, 2012; Degani, 2015, 2017). The divergence among various populations found in the present study is similar to differences as reported for *Triturus karelinii* (Farasat *et al*., 2016).

The contribution of the present study is that adding three genetic markers (rhodopsin, 12S and 16S), that were used before in this species, showed divergences among populations surviving in different ecological conditions, which might be affected by various environmental factors (Pearlson and Degani, 2011; Degani 2017). The results of this study derives support from a previous study on *Triturus v. vittatus* using RAPD (Pearlson and Degani, 2007a), and nucleotide sequences of the mitochondrial cytochrome b and the control region (Dloop) (Pearlson *et al.*, 2010). These studies on populations from similar extreme conditions is in agreement with the present study, which found that under relatively extreme conditions (i.e. Berekhya and Afeka) or in Damascus, divergences exist in other populations from more humid sites in northern Israel.

The present result is also in agreement with other Urodeles, e.g. *Salamandra infraimmaculata*, in which the genetic variation among various habitats was examined (Goldberg *et al*., 2009, 2010, 2011; Goedbloed *et al*., 2017) or the anura species, e.g. toad (*Pseudepidalea viridis*) (Degani *et al*., 2013 a). There are two newts' habitats during their life cycle, aquatic and terrestrial. Degani (2017) suggested that the adaption to terrestrial habitats affects the newts distribution more than aquatic habitats (Pearlson and Degani, 2011). The result of the present study agrees with this hypothesis, whereby the genetic divergence among habitats in this area of extreme terrestrial conditions is higher than for other populations.

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