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**Research article**

# THE ECOLOGY AND VARIATION IN DNA OF *RANA BEDRIAGAE* FROM VARIOUS BREEDING SITES IN NORTHERN ISRAEL

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## Abstract

An ecological and molecular variation study was carried out on marsh frog (*Rana bedriagae)* larvae from habitats of different locations and altitudes in northern Israel. This study included a series of field and laboratory monitorings, conducted on aquatic habitats over four years, to test the ecological conditions of the breeding habitat selected by the marsh frog. The larval growth periods differed among the populations of the various ponds, varying among the breeding sites and changing from winter to summer, throughout the years of the study. In the ponds (Lehavot and Fara), the water availability period extended from the winter until the end of the summer, at which time, the ponds dry up. The pond sizes varied, decreasing from 1000 $m^3$ to 0 $m^3$, while the volume of the spring remained relatively constant, 3-5 $m^3$. The temperatures ranged between 5 to 30 ºC, and the rates of temperature change were higher in ponds (Lehavot and Fara) than in the spring water (Navoraya). Dissolved oxygen concentrations ranged between 0-120% saturation and were higher in winter and lower in the summer. The pH in the pond was high (8-9.5) compared to that of the spring water (7.5-8), and the conductivity was relatively similar in Navoraya Spring and Fara Pond, most of the time, remaining constant from January to October. However, the conductivity in Lehavot was significantly higher, increasing dramatically during the summer.

The mitochondrial (mt) genes, *12S* and *16S*, were amplified for sequencing and the assessment of nuclear genetic variation was carried out by random amplification of polymorphic DNA (RAPD PCR). The nucleotide sequences of the DNA fragments were determined from a 367 bp clone of *12S* and a 525 bp clone of *16S*. The *12S* fragment varied at the nucleotide position 331 among populations of various breeding sites, with 'T' in the case of Lehavot Pond, Fara Pond, Navoraya Pond and Jauda Pond and 'C' in the case of Wasset Pond. The *16S* sequences differed among populations at the nucleotide positions, 4 and 230; Lehavot Pond had T and C, Wasset Pond, Fara Pond and Navoraya Pond had C and T and Jauda Pond had Y and T, respectively (GenBank accession numbers from FJ595209 to FJ595213).

The analysis of these *12S* sequences with Arlequin software demonstrated a high genetic identity among the five populations (99.7-100%). The *16S* gene sequence varied slightly among the populations of the breeding sites in Israel at two nucleotide sites, with a high genetic identity of 99.6 – 100%.

The DNA variation among *R. bedriagae* populations from various breeding sites was also assessed by RAPD PCR using the primers, OPA-4 and OPA-7. There were 5 - 10 bands when OPA-4 was used and 7 - 12 bands with OPA-7. When comparisons were made between paired populations, band sharing (BS) analysis showed a variance between 0.5 – 0.8 in PCRs using the primer OPA-4 and between 0.7 – 1.0 when using primer OPA-7. When primer OPA-4 was employed, the lowest similarity between different breeding sites was found between Navoraya Spring and Wasset Pond, and the highest similarity was between Navoraya Spring and Lehavot Pond. When using the primer OPA-7, the lowest similarity between populations of different breeding sites was between Lehavot Pond and Wasset Pond, and the highest similarity was between Lehavot Pond and Navoraya Spring.

**Key words:** *Rana bedriagae*; breeding sites, DNA variation; mitochondrial DNA

## Introduction

The species, *Rana ridibunda*, is distributed throughout central and southern Europe and western Asia, reaching Israel, the southern border of its distribution (Arikan et al., 1998). The systematics regarding *R. ridibunda* are under investigation, and synonymous names are used. In this paper, we chose to use the name, *Rana bedriagae* (Sinsch and Schneider 2002) for the frogs in Israel. This species, which was formerly represented by two subspecies, became a monotypical species after the *Rana perezi* subspecies was accepted as a separate species by Hotz (1974). Schneider et al. (1992) discovered a new species (*Rana levantina*), when studying *R. bedriagae* populations in Israel by morphological and bioacoustical means. Joermann et al. (1988) have included *R. ridibunda* populations from western Turkey among *R. levantina*, according to their bioacoustic properties. Also in Greece, some *R. ridibunda* populations have been established as two separate species (*Rana eperiotica and Rana balcanica*) by means of morphological and bioacoustical studies (Schneider et al., 1993). In Turkey, which is within the area of the distribution, the Ivriz population was included in *R. ridibunda caralitana*, according to the results of the morphological analysis. Furthermore, the distribution area of *R. r. caralitana* has been extended (Arikan et al., 1998).

Kyriakopoulolj-Sklav et al. (2008) studied the morphological and genetic differentiation among three populations of the *R. ridibunda* from northern Greece, using selected morphometric characters and electrophoresis of polymorphic enzymes. The overall pattern was interpreted as a response to different selection regimes within the morphometric phenotype (including the presence of a syntopic congener), absent in the allozymes. Plotner et al. (2008) stated that Anatolian marsh frogs do not represent *R. bedriagae*, according to mitochondrial (mt) DNA techniques. He described the relationship between *R. ridibunda* and *Rana lessonae* in Europe (Plotner et al., 2008). Natural primary hybridizations between *R. ridibunda* and *R. lessonae* DNA occur (Berger, 1970; Borkin et al., 1979.).

The taxonomic reassessment of Middle Eastern water frogs include an Israeli study by Sinsch and Schneider (2002). Eight morphometric features of water frogs of 14 localities in Turkey, Syria, Jordan and Israel were compared with those of *R. ridibunda* in Kazakhstan, Armenia and Greece (Thrace). The study sites included the typical localities of *R. ridibunda*, *R. r. caralitana*, *Rana esculenta* var. *bedriagae* and *R. levantina*. Multivariate comparisons (principal-component analysis, discriminant analysis), based on the $\log_{10}$-transformed variables, demonstrate that the data set includes only two taxa that differ significantly in size and shape. By applying a morphospecies criterion, *R. ridibunda* was found to be represented exclusively by the three reference populations, whereas all other populations (in Turkey, Syria, Jordan and Israel) represent the same species, *R. bedriagae*. Data collected on *R. bedriagae* (*ridibunda*) in northern Israel and on other species of amphibians have been published in various papers (Degani, 1982; Degani, 1986; Degani and Kaplan, 1999). The structure and mating call variations of *R. bedriagae* in Israel were studied by Nevo and Schneider (1983), and the genetic differentiation between Israeli and Greek populations were documented by Nevo and Filippucci (1988).

Little information has been described on ecology and life cycle of *R. bedriagae* in Israel (Degani, 1982; Degani, 1986; Degani and Kaplan, 1999). Ecological and biological conditions of breeding sites inhabited by amphibian larvae in northern Israel, including winter ponds, rock pools, springs and streams, have been investigated (Degani, 1982; Degani and Kaplan, 1999). The breeding season is in May and June, but some larvae may be found in the water throughout the summer and may complete metamorphosis at the end of the summer or at the beginning of the winter. However, not much information has been published on the ecological conditions of the

water bodies where *R. bedriagae* exist and breed. The present study tests the ecological conditions in the breeding water bodies and the genetic differences in the populations of *R. bedriagae* in northern Israel by means of random amplification of polymorphic DNA (RAPD PCR) and by the analysis of *R. bedriagae* mt DNA.

## Materials and Methods

### Sites studied and larvae collected

The sites selected to be studied included a range of amphibian aquatic breeding habitats. All main types of aquatic habitats populated by amphibian larvae in northern Israel were investigated, according to the procedure described by Degani and Kaplan (1999). Larvae were collected randomly by a hand net (Degani and Mendelssohn, 1983), and their species were identified. The five breeding sites of frog of this study, include four winter pools and spring, were examined over four consecutive years (2005–2008). Their locations and descriptions are presented in figure 1 and table 1, respectively. All larvae were grouped according to the specific water body in which they had been observed. Only the sites in which *R. bedriagae* larvae were found, are described in the present study (Fig.1).

### Biotic and abiotic parameters of the water

Water quality was determined as described previously by Pearlson and Degani (2007). Water parameters were measured every two weeks during the period in which the pools were filling up. *In situ* temperature and dissolved oxygen data were obtained by a hand-held oxygen meter (WTW, Oxi330 set, Germany). Parameters were analyzed by one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls (SNK) test, using Graph-Pad Prism software (Graph Pad, San Diego, CA). The level of significance between groups was set at $p < 0.05$ (ANOVA).

### Specimens

In order to analyze and characterize *R. bedriagae* populations from various areas in northern Israel, ten specimens of *R. bedriagae* were collected from each population. The tissues of the specimens were studied by mitochondrial sequence analysis.

### 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 employs 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 NanoDrop100 (ThermoFisherScientific, Wilmington, DE, USA).

DNA of *R. bedriagae*, *12S* and *16S*, was amplified by PCR and analyzed by RAPD PCR. Primers for DNA amplification of *12S* genes were based on those of Veith et al. (1992). 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 gemonic 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 were sequenced at the Hy Laboratories (Rehovot, Israel).

### DNA sequence analysis

The primer decamers (Mikulicek and Pialek, 2003), OPA-4 (5' AATCGGGCTG) and OPA-7 (5'GAAACGGGTG), were found to be best for the sequence analysis of *R. bedriagae* by RAPD PCR. The reaction 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 ng gemonic 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.

DNA of every population was first run on one gel to determine if there were differences within the population. Subsequently, the DNA of the populations was run on another gel (one gel) to compare patterns among 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* (Jeffreys and Morton, 1987; Wetton et al., 1987). The PCR patterns were compared only between samples that had been run on a single gel. Differences in BS were examined by the *d*-test for differences between proportions (Parker, 1976). 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 the multiple sequence alignment.

## Results

Larval growth of the *R. bedriagae* is presented in figure 2. The larval growth periods differed among the various ponds and over the years of the study. In the ponds, the larval growth periods occurred during the summer from May to September. Only in spring water (Navoraya) the larvae found all year round (Fig. 2). The growth of larvae differed in various breeding sites, varying from winter to summer. Larval growth was calculated to be: $Y = 0.9X + 3.1$, $R^2 = 0.9854$ in Navoraya Spring in the winter and $Y = 0.5889e^{0.2488x}$, $R^2 = 0.9827$ in the summer; $Y = 0.1244X^{1.6984}$, $R^2 = 0.9827$ in Fara Pond in the summer; and $Y = -X^2 + 11.9X - 31$, $R^2 = 1$ in Lehavot Pond in the summer (Fig. 2).

The water quality parameters of three different ponds containing larvae of *R. bedriagae* are shown in figure 3. In the ponds (Lehavot and Fara), the period during which water was available was from the winter to the end of the summer, when the ponds dry up. The spring water was available all year round (Fig. 3). The size of the ponds changed from 1000 $m^3$ to 0 $m^3$, while the spring volume remained between 3 to 5 $m^3$. The temperatures in the various water bodies increased significantly from winter to spring and into the summer, with temperatures ranging between 5 to 30 ºC (Fig. 3). The rate of the temperature change was higher in the ponds (Fara and Lehavot) than in the spring water (Navoraya). However, no significant differences were found among the temperatures of the various water bodies during the four years studied (ANOVA, F-value=2.48; P = 0.11 >0.05).

Dissolved oxygen concentrations ranged between 0-120% saturation and were high in winter, decreasing during the summer. A greatly significant difference (ANOVA, F-value=9.24; P = 0.001 <0.05) in oxygen concentration was found between the spring water and ponds, only during the winter period (December to March) (Fig. 3). The pH in the pond was significantly higher (8-9.5) compared to that of the spring water (7.5-8) (ANOVA, F-value=14.72; P = 0.00006 <0.05) (Fig. 3). The conductivity was relatively similar most of the time, remaining constant in the spring water and in the Navoraya and Fara ponds from January to October. However, the conductivity in Lehavot was significantly higher (ANOVA, F-value=11.5; P = 0.00024 <0.05) and increased dramatically during the summer (Fig. 3). The water volume of the pond varied and decreased in the summer at the time when the ponds dry up (Fig. 3). However, the water volume of the spring was relatively constant.

The nucleotide sequences of the DNA fragments were determined from a 367 bp clone of *12S* and a 525 bp clone of *16S* (GenBank accession numbers from FJ595209 to FJ595213, Table 2). The *12S* fragment varied at nucleotide position 331 among populations of various breeding sites; in Wasset Pond they had a C, while in Lehavot Pond, Fara Pond, Navoraya Pond and Jauda pond, they had a T.

The *16S* sequence varied among populations of the ponds at nucleotide positions 4 and 230. In Lehavot Pond, the positions were occupied by T and C, in Wasset Pond, Fara Pond and Navoraya Pond, by C and T and in Jauda Pond, by Y and T, respectively (Table 2).

A similar situation was revealed when comparing the *12S* gene variation among populations from various breeding sites (Fig. 4). The analysis of five sequences with Arlequin software demonstrated a high gene identity (99.7-100%).

The *16S* gene sequence varied among populations of breeding sites in Israel at two nucleotide sites (Fig. 5). Again, there was a high genetic identity among the populations of the various breeding sites (99.6 – 100%).

The DNA variation among *R. bedriagae* populations at various breeding sites was assessed using the primers, OPA-4 and OPA-7, as presented in figure 6. There were 5 - 10 bands when OPA-4 was used and 7 - 12 bands with OPA-7. When comparisons were made between paired populations, as in Table 3, the BS varied between 0.5 – 0.8 in PCRs with primer OPA-4 and between 0.7 – 1.0, when using primer OPA-7. The lowest similarity between populations of different breeding sites, when using primer OPA-4, was detected between Navoraya Spring (D) and Wasset Pond (B) and the highest similarity was revealed to be between Navoraya Spring (D) and Lehavot Pond (A). When using primer OPA-7, the lowest similarity between populations of different breeding sites was found between Lehavot Pond (A) and Wasset Pond (B) and the highest similarity was discovered to be between Lehavot Pond (A) and Navoraya Spring (D ).

## Discussion

In this study, it was demonstrated that the distribution of *R. bedriagae* among the breeding sites was related to biotic and abiotic factors of the aquatic and terrestrial habitat, but not to the type of water body. We found that among the various biotic and abiotic parameters, temperature was a major factor. Freidenburg, and Skelly (2008) reported data supporting their hypothesis that the wood frog (*Rana sylvatica*) populations undergo localized selection leading to counter gradient patterns of thermal preference behavior.

A large number of environmental variables were examined in the present study to characterize the water quality at the breeding sites, due to the difficulty in foreseeing factors that may influence the selection of a certain water body as a breeding habitat. The range in water qualities of sites occupied by larvae of various amphibians was very narrow. Degani and Kaplan (1999) examined forty one amphibian habitats of various types in northern Israel, and only in eight of them found larvae of *R. sylvatica* (*bedriagae*). Only in one winter pond habitat were the ecological parameters of the water studied in detail (Degani, 1982; Degani, 1986), and the results are in agreement with this study. They showed that larvae existed in unpredictable breeding sites, such as the winter pond, during the spring and summer only if water was available. In the present investigation, the larvae were detected even in spring water, in which the water temperatures were very similar during the winter and summer. These results support the hypothesis that the larvae can grow and complete metamorphosis at temperatures above 20 ºC. However, larvae may be found in permanent water bodies, such as the spring, in the winter, as well. Apparently, spawning was carried out in the summer, but the larvae grew slowly at relatively low temperatures below 20 ºC, requiring a longer time to complete metamorphosis. In water bodies where the temperatures are below 18 ºC all year round, larvae of *R. sylvatica* were not observed (Goldberg et al. 2009a). The breeding season of *R. sylvatica* in Israel is spring and the beginning of summer in winter ponds. However, larvae may be discovered in spring water, not only during the spring and summer, but also in the winter, as was demonstrated in the present study. Another water quality, monitored in this study, was the oxygen concentration, which behaved reciprocally to the temperature and pH. Conductivity was relatively constant in the spring water compared to in the winter ponds, supporting previous reports (Goldberg et al. 2009a).

Different methods have been used to study the variation among populations of the genus *Rana* in order to understand the taxonomy, evolution and ecological aspects of this genus. The most popular methods employed to study the *Rana* species have been morphology (Arikan et al., 1998; Hotz, 1974; Kyriakopoulou-Sklavounou et al., 2008; Schneider et al., 1992; Sinsch and Schneider, 2002; Tarih, 1999), bioacoustics (Joermann et al., 1988; Nevo and Schneider, 1983; Schneider et al., 1992), electrophoresis of polymorphic enzymes (Kyriakopoulou-Sklavounou et al., 2008) and mt DNA techniques (Plotner et al., 2008). Most of the studies compared various species that were distributed in relatively large geographical areas. In the present study, the mt DNA and genomic DNA variation were examined in various populations of one species (*R. bedriagae*) in a

relatively small area. The results indicate that the variation among these various populations was very low according to the mt DNA (*12S* and *16S*), as compared to the variation found in the genomic DNA (when using the RAPD PCR method).

In northern Israel, the *R. sylvatica* exists around relatively permanent water bodies, where water is available throughout the year (Degani and Kaplan, 1999). Its reproduction period is in the summer (Degani, 1982; Degani, 1986). The conditions at the breeding sites were relatively constant, and low genetic variation was found in the various habitats examined in the present study. Apparently, DNA variations are higher among populations of other amphibian species in Israel that use more unpredictable habits for breeding than *R. sylvatica*. Pearlson and Degani (2008) studied the life cycle of *Triturus v. vittatus* that used unpredictable habitats (winter ponds) at localities of various altitudes in Israel, ranging from 212 to 740 m above sea level (ASL). The genetic variation among *T. v. vittatus* populations seemed to be higher than that of *R. bedriagae*, according to mt DNA sequences of cytochrome *b* and control genes (Pearlson et al., 2009). In addition, results of the analysis of the cytochrome *b* and control gene sequences demonstrated that the mtDNA genetic variation among different populations of *Salamandra infraimmaculata* larvae in northern Israel was higher than the mt DNA variation among *R. bedriagae* populations. Results regarding both species, *T. v. vittatus* and *S. infraimmaculata*, agree with those of the this study, which indicate that the RAPD method is more sensitive and suitable in the study of genetic variation among different populations in the same geographical region (Degani et al., 1999; Goldberg et al., 2007; Pearlson et al., 2009; Pearlson and Degani, 2007).

Ethologically, bioacoustic analysis of *R. sylvatica* mating calls in Israel (Nevo and Schneider, 1983) Thus, it was concluded that *R. sylvatica* is not homogeneous within its range of distribution. On the other hand, Joermann et al. (1988) found a close relationship between *R. ridibunda* in Israel and Turkey, using bioacoustic analysis. Nevo and Yang (1982) investigated the *R. sylvatica* in Israel in diverse ecologies from the northern Mediterranean to the Judean Desert. Based on a 28 gene loci electrophoretical test, they found a high identity among the populations (mean D = 0.028, genetic identity = 0.781), in contrast to the genetic difference revealed between Israeli and Greek *R. sylvatica* populations, using 41 isozyme gene loci (D = 0.247) (Nevo and Filippucci, 1988).

## Conclusion

The breeding season of *R. sylvatica* in Israel is spring and the beginning of summer in winter ponds. However, larvae may be discovered in spring water, not only during the spring and summer, but also in the winter, as was demonstrated in the present study. These results correspond with those of the present study, which by two different methods, demonstrated that there is a very high genetic identity among the populations of *R. sylvatica*, distributed in a small geographical range.

## LITERATURE CITED

[1] Arikan, H., K. Olgun, I. E. Evik, and C. Tok. 1998. A Taxonomical Study on the Rana ridibunda PALLAS, 1771 (Anura: Ranidae) Population from Ivriz-Eregli (Konya). Turkish Journal of Zoology 22: 181-184.

[2] Berger, L. 1970. Some characteristics of the crosses within Rana esculenta complex in postlarval development. Annales Zoologici Fennici 27: 373–416.

[3] Borkin, L. J., W. I. Garanin, N. T. Tichenko, and I. A. Zaune. 1979. Some results in the green frogs survey in the USSR. Mitteilungen aus dem Zoologischen Museum in Berlin 55: 153–170.

[4] Degani, G. 1982. Amphibian tadpole interaction in winter pond. Hydrobiologia 96: 3-7.

[5] Degani, G. 1986. Growth and behavior of six species of amphibian larvae in winter pond in Israel. Hydrobiologia 140: 5-10.

[6] Degani, G., K. Jackson, C. Dosoretz, and Y. Plotzky. 1999. Molecular DNA variation in Salamandra s. infraimmaculata from different habitats. Israel Journal of Zoology 44: 239-246.

[7] Degani, G., and D. Kaplan. 1999. Distribution of amphibian larvae in Israeli habitats with changeable water availability. Hydrobiologia 405: 49-56.

[8] Degani, G., and H. Mendelssohn. 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.

[9] Freidenburg, L. K., and D. K. Skelly. 2008. Microgeographical variation in thermal preference by an amphibian. Ecology.Letters 7: 369 - 373.

[10] Goldberg, T., O. Pearlson, E. Nevo, and G. Degani. 2007. Mitochondrial DNA analysis of Salamandra infraimmaculata larvae from habitats in northern Israel. Progrese şi Perspective in Medicina Veterinară" - Lucrări ştiinţifice 50: 23-31.

[11] Hotz, H. 1974. Problem aus vielen Frageneuropaeische Gr□nfr□sche (Rana esculenta-Komplex) und ihre Verbreitung. Natur u Museum 104: 262-272.

[12] Jeffreys, A. J., and D. B. Morton. 1987. DNA fingerprints of dogs and cats. Animal Genetics 18: 1-15.

[13] Joermann, G., I. Baran, and H. Schneider. 1988. The mating call of Rana ridibunda (Amphibia: Anura) in Western Turkey: Bioacoustic analysis and taxonomic consequences. Zoologischer Anzeiger 220: 225-232.

[14] Kyriakopoulou-Sklavounou, P., P. Sjogren, and H. Jansson. 2008. Discordant genetic and morphological differentiation between three Rana ridibunda populations from northern Greece. Hereditas 115: 51-61.

[15] Mikulicek, P., and J. Pialek. 2003. Molecular identification of three crested newt species (Triturus cristatus superspecies) by RAPD markers. Amphibia-Reptilia 24: 201-207.

[16] Nevo, E., and M. G. Filippucci. 1988. Genetic differentiation between Israeli and Greek population of the marsh frog, Rana ridibunda. Zoologischer Anzeiger 221: 418-424.

[17] Nevo, E., and H. Schneider. 1983. Structure and variation of Rana ridibunda mating call in Israel (Amphibia: Anura). Israel Journal of Zoology 32: 45-60.

[18] Nevo, E., and S. Y. Yang. 1982. Genetic diversity and ecological relationship of marsh frog populations in Israel. Theory of Applied Genetics 68: 317-330.

[19] Parker, R. 1976. Introduction of Statistics for Biology. Camelot Press, Southampton, UK.

[20] Pearlson, O., L. Blaustein, S. Snir, D. Goldberg, and G. D. . 2009. Molecular variation in Triturus vittatus vittatus (Urodela) from breeding sites near the southern limit of its distribution inferred from mitochondrial DNA sequence of cytochrome b gene and control Region. Current Herpetology: submitted for publication.

[21] Pearlson, O., and G. Degani. 2007. Molecular DNA variations among Triturus vittatus vittatus (Urodela) from different breeding sites at the southern limit of its distribution. Acta Herpetologica 2: 69-77.

[22] Pearlson, O., and G. Degani. 2008. The life history of Triturus v. vittatus (Urodela) in various habitats. Asiatic Herpetolological Research 11: 91–95.

[23] Plotner, J. et al. 2008. Widespread unidirectional transfer of mitochondrial DNA: a case in western Palaearctic water frogs. Journal of Evolutionay Biology 21: 668-681.

[24] Schneider, H., U. Sinsch, and E. Nevo. 1992. The lake frogs in Israel represent a new species. Zoologischer Anzeiger 228: 97-106.

[25] Schneider, H., U. Sinsch, and T. S. Sofianidou. 1993. The water frogs of Greece. Bioacoustic evidence for a new species. Zeitschrift fur Zoologische Systematik und Evolutions Forschung 31: 47-63.

[26] Sinsch, U., and H. Schneider. 2002. Taxonomic reassessment of Middle Eastern water frogs: Morphological variation among populations considered as Rana ridibunda, R. bedriagae or R. levantina. Journal of Zoological Systematics and Evolutionary Research 37: 67 – 74.

[27] Tarih, G.-. 1999. Morphological investigation of Rana ridibunda (Anura: Ranidae) populations in the Black Sea region. Turkish Journal of Zoology 23: 801-806.

[28] Veith, M. 1992. The fire salamander Salamandra salamandra L. in central Europe: subspecies, distribution and intergradation. Amphibia-Reptilia 13: 297-313.

[29] Wetton, J. H., R. E. Carter, D. T. Parkin, and D. Walters. 1987. Demographic study of a wild house sparrow population by DNA fingerprinting. Nature. 327: 147-149.

## FIGURES

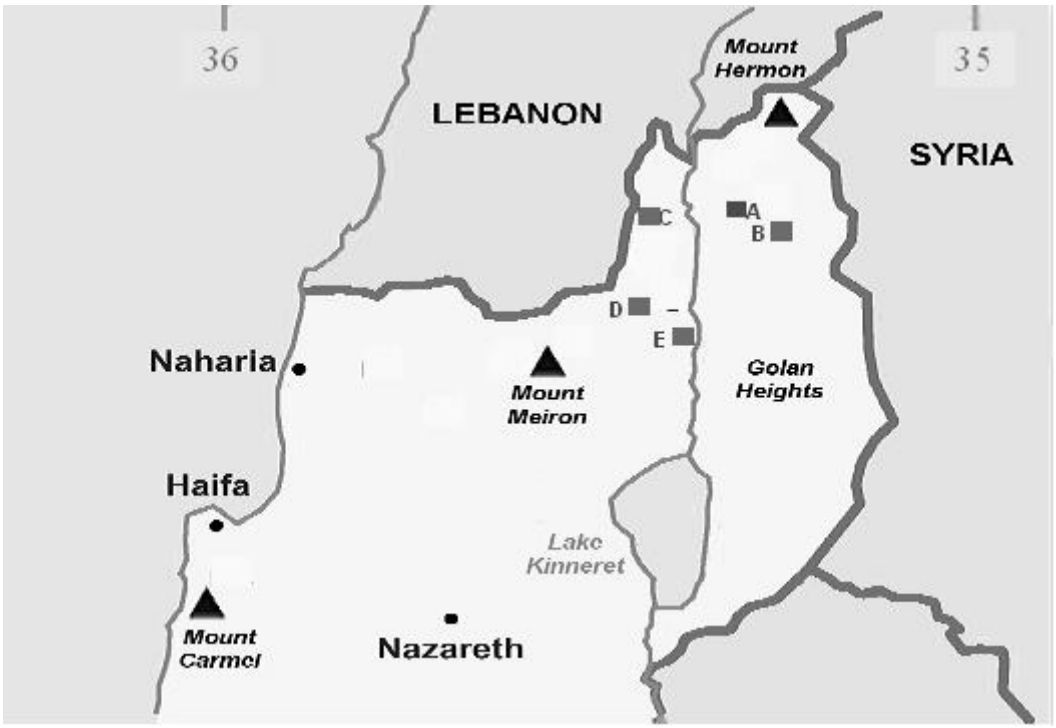



**Figure 1:** A - Lehavot Pond, B – Wasset Pond, C – Fara Pond, D – Navoraya Spring, E – Jauda Pond.

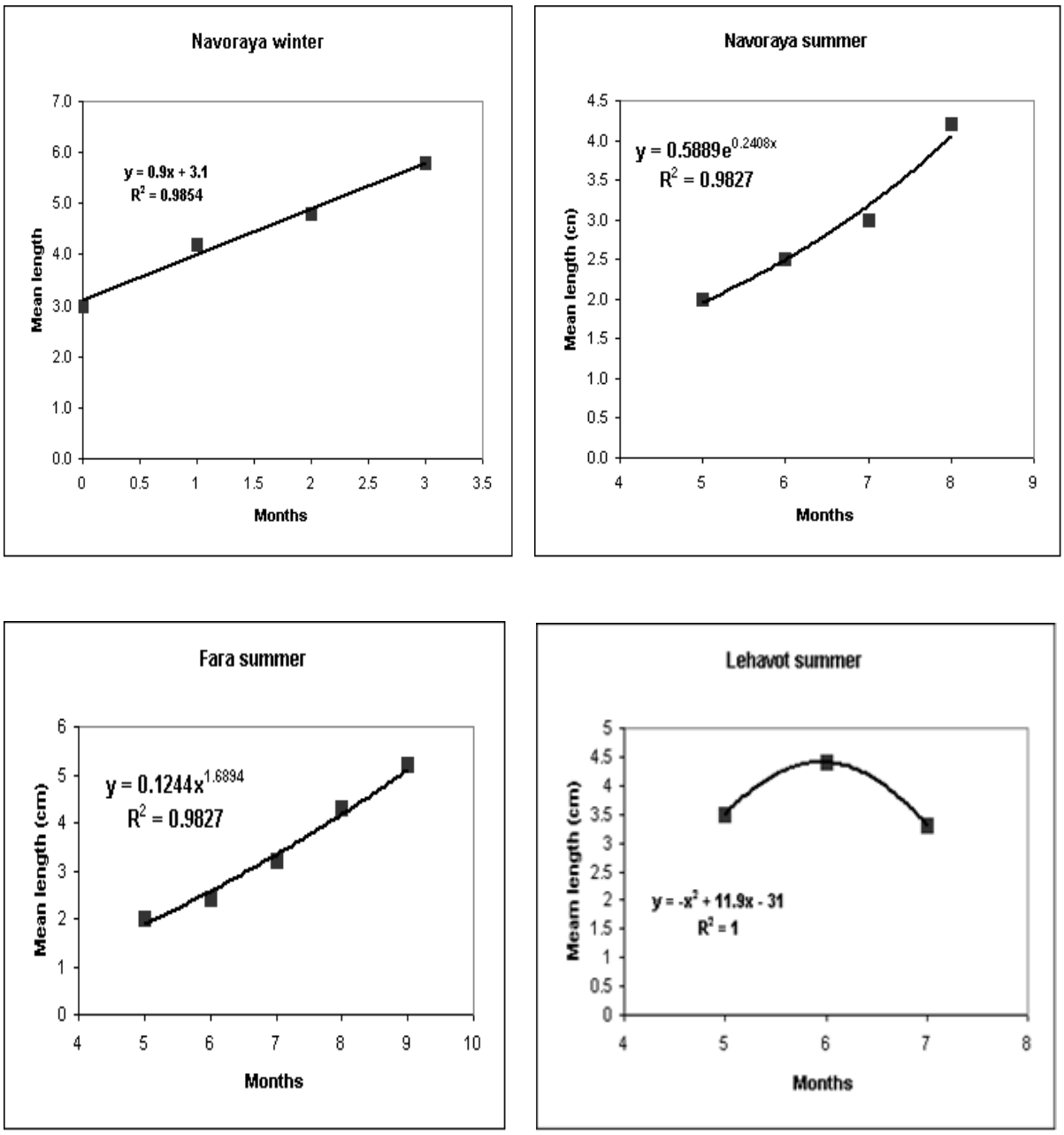



**Figure 2:** Larval growth of *R. bedriagae* at different breeding sites.

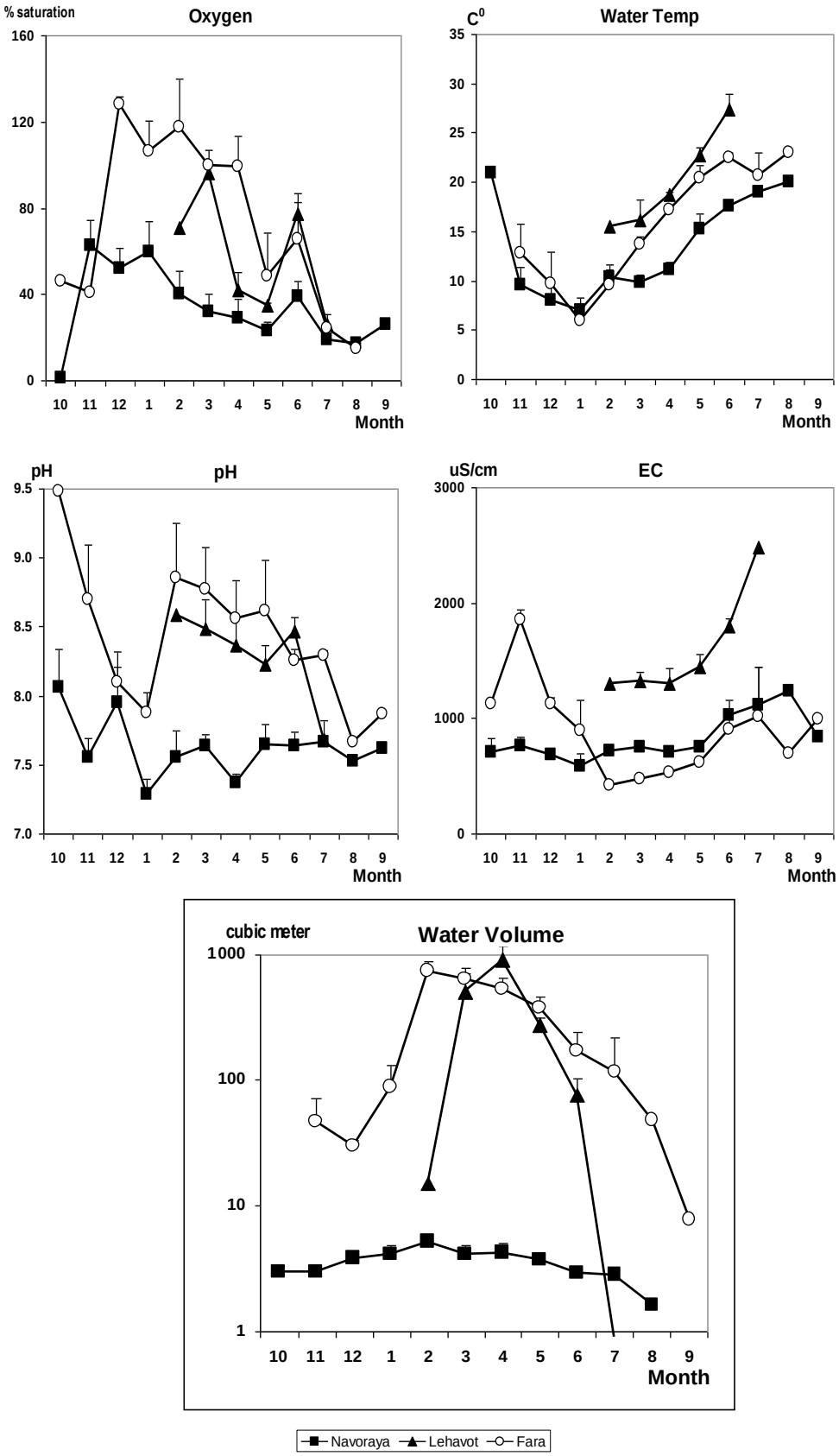
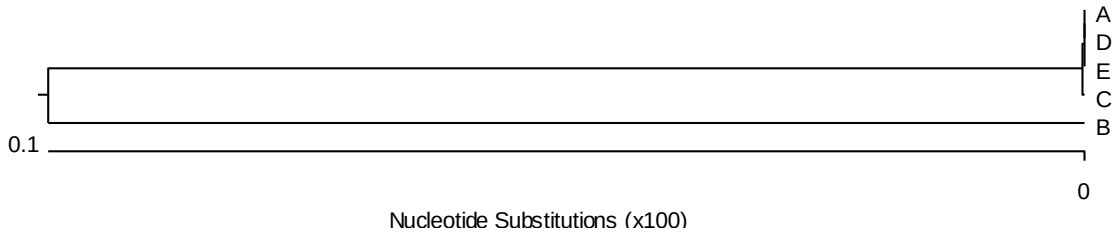



**Figure 3:** The abiotic parameters of the water at various breeding sites where larvae grow and complete metamorphosis.

a)

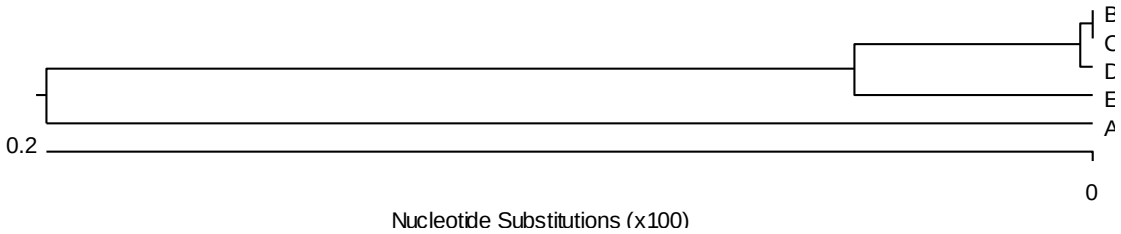



b)

| | B | C | E | A | D |
|---|---|---|---|---|---|
| **B** | - | **99.7** | **99.7** | **99.7** | **99.7** |
| **C** | - | - | **100.0** | **100.0** | **100.0** |
| **E** | - | - | - | **100.0** | **100.0** |
| **A** | - | - | - | - | **100.0** |
| **D** | - | - | - | - | - |

**Figure 4:** Calculated standard parameters of nucleotide variation in the mitochondrial (mt) gene, *12S*, among populations. The evolutionary history was inferred using the neighbor-joining method.

a)




b)

| | B | C | E | A | D |
|---|---|---|---|---|---|
| **B** | - | **100.0** | **99.8** | **99.6** | **100.0** |
| **C** | - | - | **99.8** | **99.6** | **100.0** |
| **E** | - | - | - | **99.6** | **99.8** |
| **A** | - | - | - | - | **99.6** |
| **D** | - | - | - | - | - |

**Figure 5:** Calculated standard parameters of nucleotide variation in the mitochondrial (mt) gene, *16S*, among populations. The evolutionary history was inferred using the neighbor-joining method.

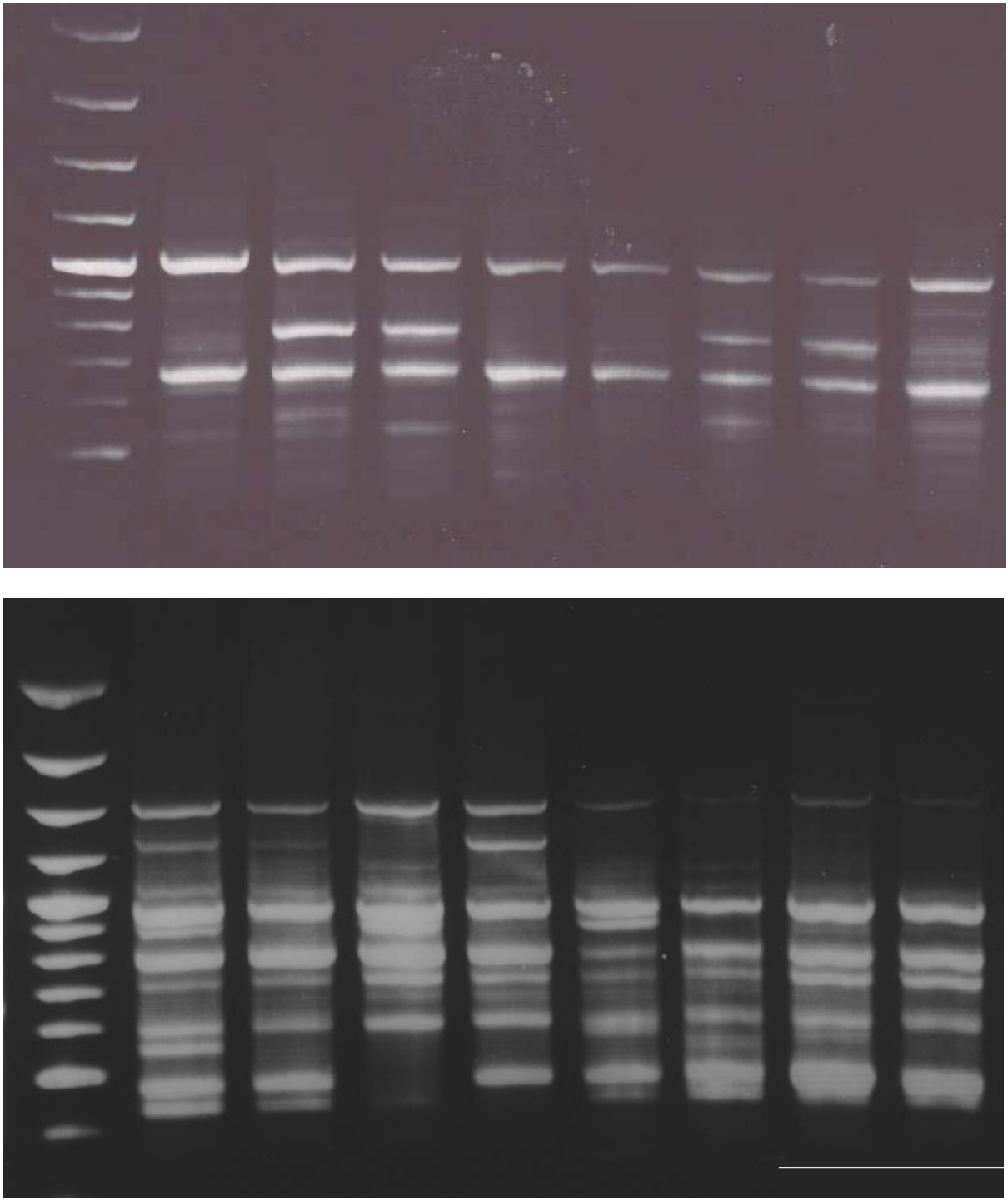


**Figure 6:** RAPD PCR results using the OPA-4 and OPA-7 primers. A - Lehavot Pond, B – Wasset Pond, C – Fara Pond, D – Navoraya Spring, E – Jauda Pond.

**Table 1:** Breeding sites studied

| | **Latitude** | **Longitude** | ***Altitude (m ASL)*** |
|---|---|---|---|
| A - Lehavot_Pond | 284300 | 210150 | 70 |
| B - Wasset Pond | 282800 | 219000 | 850 |
| C - Fara Pond | 236119 | 157022 | 446 |
| D - Navoraya Spring | 274808 | 192873 | 665 |
| E - Jauda Spring | 259700 | 205500 | 110 |

**Table 2:** The accession numbers of the sequences of *Rana bedriagae* 12S and 16S

| **Breeding Sites** | **12S** **GenBank acc. no.** | **16S** **GenBank acc. no.** |
|---|---|---|
| A - Lehavot_Pond | FJ595206 | FJ595211 |
| B - Wasset_Pond | FJ595208 | FJ595213 |
| C - Fara_Pond | FJ595204 | FJ595209 |
| D - Navoraya_Spring | FJ595207 | FJ5952127 |
| E - Jauda_Pond | FJ595205 | FJ595210 |