Acta Herpetologica 2(2): 69-77, 2007
ISSN 1827-9643 (online)

Molecular DNA variation among *Triturus vittatus vittatus* (Urodela) from different breeding sites at the southern limit of its distribution

Oren Pearlson[1, 2, 3], Gad Degani[1, 2]

[1] *MIGAL–Galilee Technology Center, P. O. Box 831, Kiryat Shmona 11016, Israel.* Corresponding author. Gad Degani, E-mail: gad@migal.org.il
[2] *School of Science and Technology, Tel Hai Academic College, Galilee, Israel*
[3] *Institute of Evolution, Faculty of Sciences and Science Education, University of Haifa, Israel*

Abstract. Molecular DNA variation among *Triturus vittatus vittatus* (striped newt) from different breeding sites at the southern limit of the species distribution (where environmental conditions are most extreme) was studied by the random amplification of polymorphic DNA (RAPD) method that has been found to be appropriate for other *Triturus* species. Altitudes of the localities ranged between 15-740 m a.s.l. Of the 20 primers employed, OPA-16 was the only one suitable for *T. vittatus,* revealing a different band pattern for different populations. Genetic similarity was calculated by band sharing, which demonstrated a high similarity among the Israeli populations.

Keywords. Genetic polymorphism, xeric habitat, conservation, extreme conditions, *Triturus vittatus vittatus*.

INTRODUCTION

The banded newt, *Triturus vittatus vitattus*, is an endangered species in Israel (Geffen et al., 1987). Other species and subspecies of this genus are endangered in other regions of the world. *Triturus v. vitattus* is distributed throughout the western Caucasus, Turkey, Lebanon, Syria, Israel, Iraq and perhaps, Jordan (Borkin et al., 2003; Litvinchuk et al., 2005). The biology and life cycle of *Triturus vittatus* in Europe and the Mediterranean region have been described by Raxworthy (1989) and Olgun et al. (1997). Until recently, *Triturus vittatus* was thought to have two subspecies: *Triturus 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. Based on its trunk vertebrae count, genome size and allozyme data, Litvinchuk et al. (2005) suggested that the northern taxon, *T. v. ophryticus*, is subdivided into two geographic fragments,

the "western group", with populations from western Anatolian Turkey, and the "eastern group", composed of populations from the remaining region of Pontic Turkey and the western Caucasus.

At the southern limit of *T. v. vittatus* distribution (in Israel), environmental conditions are the most extreme. There, limiting factors are likely to be the breeding sites and dryness of the terrestrial habitat. The biology and life cycle of *T. v. vittatus* in northern Israel and the Upper Galilee have been described by Degani and Mendelssohn (1983) and Degani (1986a, 1996), while a population in central Israel has been studied by Geffen et al. (1987). *Triturus v. vittatus* inhabit mainly winter pools that typically contain water until the beginning of summer, although occasionally they have water all year-round (Degani and Kaplan, 1999). Like other newts, *T. v. vittatus* requires water bodies surrounded by an adequate terrestrial habitat to support both life phases. If either habitat is damaged, a population may be unable to survive.

Zajc and Arntzen (2000) utilized fragments of mitochondrial DNA to study evolutionary aspects of *Triturus*. Their findings support the sister-taxon relationship of *T. vulgaris* and *T. montadoni* and the position of *T. vittatus* within the subgenus, *Triturus,* as a sister taxon of the clade of the large-bodied newts, *T. marmoratus* and *T. cristatus*. According to the allozyme analysis of Litvinchuk et al. (2005), the genus *Triturus* is not a monophyletic taxon and representatives of the genus *Neurergus* are closely related to *T. karelinii*. Based on these results and reports from earlier studies on mitochondrial DNA analyses in newts by other authors, in which it was demonstrated that the genus *Triturus* is paraphyletic, a taxonomic scheme was proposed wherein the genus *Triturus* was divided into four monophyletic genera, *Triturus* s.s., *Lophinus*, *Mensotriton* and *Ommatotriton*. Furthermore, the substantial genetic differences that was found between the northern and southern samples of *T. vittatus*, together with the unique distribution, external morphology and coloration differences of the latter, led them to suggest that the northern populations of *T. vittatus* be designated a distinct species, *T. ophryticus.* Mikulicek and Pialek (2003) used RAPD PCR analysis to elucidate the difference between the crested newt species, *T. cristatus*, *T. dobrogicus*, and *T. carnifex*, allowing identification of genetic relatedness among populations of different breeding sites, as an indication of the degree of isolation between the sites, an important aspect for developing management strategies to protect the species. Weisrock et al. (2006), in their study, resolved a non-monophyletic history for *Triturus*. They divided *Triturus* species into four main parts: a clade of the *T. cristatus* species, the *T. vulgaris* and *T. boscai* clade, the *T. alpestris* clade and the *T. vittatus* clade, whose sister taxon is *Neurergus*. Their findings strongly support previous studies, in which the Mediterranean island *Euproctus* species, *E. montanus* (Corsica) and *E. platycephalus* (Sardinia), form a strongly supported clade, as well. Beebee et al. (1999) used molecular techniques in order to identify three putative newt hybrids of the two parent species, *T. vulgaris* and *T. helveticus*. RAPD analysis provided straightforward and reliable proof, producing consistent results with specimens from widely separated parts of their geographic ranges. Hybrids were identified by amplification of both the mitochondrial cytochrome *b* and ATPase genes, including partial sequencing of the latter. One hybrid had a *T. vulgaris* mother, while the other two had *T. helveticus* mothers.

Much research has focused on the genetic variables of Urodela in different geographical regions (e.g. Gibbs et al., 1998; Steinfartz et al., 2000; Lecis and Norris, 2004; Riberon

et al., 2004). However, genetic variation among populations in natural habitats around breeding sites located relatively near each other has received little attention (Lecis and Norris, 2004). Various methods have been employed to study genetic variation in amphibians, including albumins in serum (Degani, 1986b), isozymes (Veith et al., 1992), RAPD PCR (Degani et al., 1999; Mikulicek and Pialek, 2003) and mitochondrial DNA sequencing (Weisrock et al., 2001).

The present study, by means of RAPD PCR, examines genetic differences, among *T. v. vittatus* populations in five different breeding sites in the southern-most region of its distribution, where environmental conditions are most extreme.

MATERIAL AND METHODS

Study sites

Nahalit Ponds (Figs. 1B, 2b): these two ponds are located side by side in cattle grazing land in the Upper Galilee (longitude 243657, latitude 776401) 665 m a.s.l. The ponds fill up with water

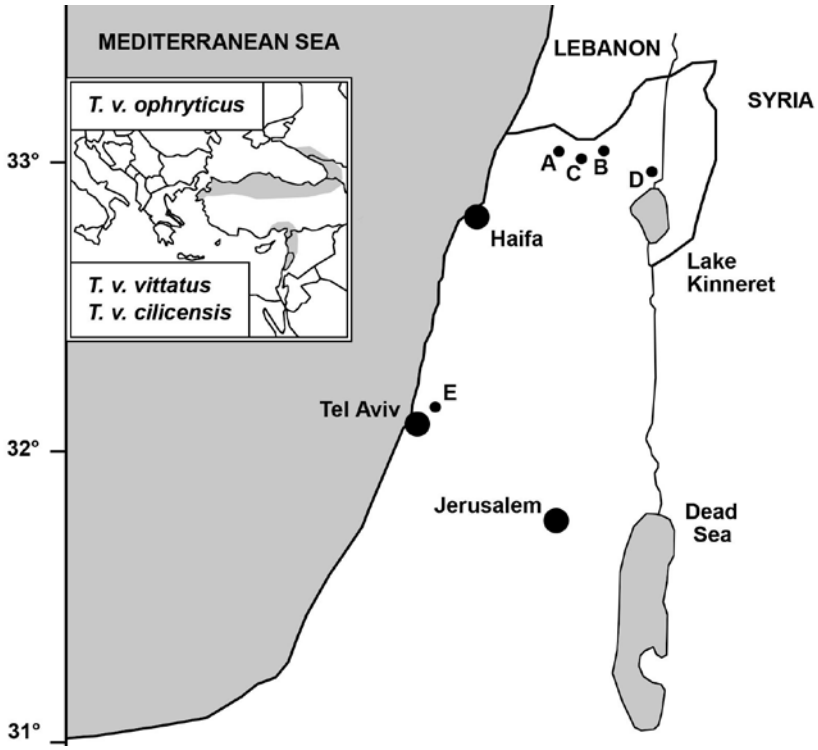

Fig. 1. The five ponds colonized by newts examined in the study: (A) Dovev Pond, (B) Nahalit Pond, (C) Matityahu Q. Pond, (D) Amiad water holes, (E) Afeka.

that seeps through chicken farms and cattle pens. The smaller pond holds water from January to May, has an area of ca 50 m^2 and a depth in the center of ca 2 m. The other one has an area of ca 1000 m^2, a depth in the center of ca 0.8 m and contains water from January to June. Both have water plants, such as *Ranunculus aquatilis*, *Scirpus tuberosus* and *Scirpus tabernae-montanus*. Adult *Triturus* inhabit the ponds from mid-January until the beginning of April, and larvae were found between mid-March and June, when the pond dries up.

Dovev Pond (Figs. 1A, 2a): the pond is a nature reserve site in the Upper Galilee (longitude 239158, latitude 772801) at an altitude of 740 m a.s.l. The pond is located among apple orchards with water from December to July. It has an area of ca 300 m^2, a depth in the center of ca 1.5 m and water plants, such as *Ranunculus aquatilis*, *Scirpus tuberosus* and *Scirpus tabernae-montanus*. Adult *Triturus* can be observed from mid-December until the beginning of April, while larvae can be discerned from mid-March until July, when the pond dries up.

Amiad water hole (Figs. 1D, 2d): The water hole is man made and is located in the southern part of the Upper Galilee (longitude 251721, latitude 757994, altitude: 212 m a.s.l.). The pond is located in cattle grazing land and holds water from December to July. The water hole has a radius of 0.7 m, an area of ca 3 m^2, a depth in the center of ca 1.5 m and water plants, such as *Ranunculus aquatilis*. Adult *Triturus* can be found in the water from mid-December until the beginning of April, while larvae can be detected only after the water has dried up in July.

Matityahu Quarry Pond (Figs. 1C, 2c): The pond in the Matityahu Quarry is located among apple orchards, in the Upper Galilee (longitude 242783, latitude 774855, altitude: 670 m a.s.l.). The pond contains water from January to June. It is ca 200 m^2 and ca 2 m deep in the center. The pond has been dug several times in the last few summers, fills up at the beginning of January and contains water until mid-June. No water plants populate the pond. Adult *Triturus* were observed from January until the beginning of April, and larvae were found from mid-March until June, when the pond dries up.

Afeka Pond (Figs. 1E, 2e): the pond is located in the central coastal plain, (longitude 182364, latitude 670453) with a mean altitude of 15 m a.s.l. The pond is situated alongside a section of a

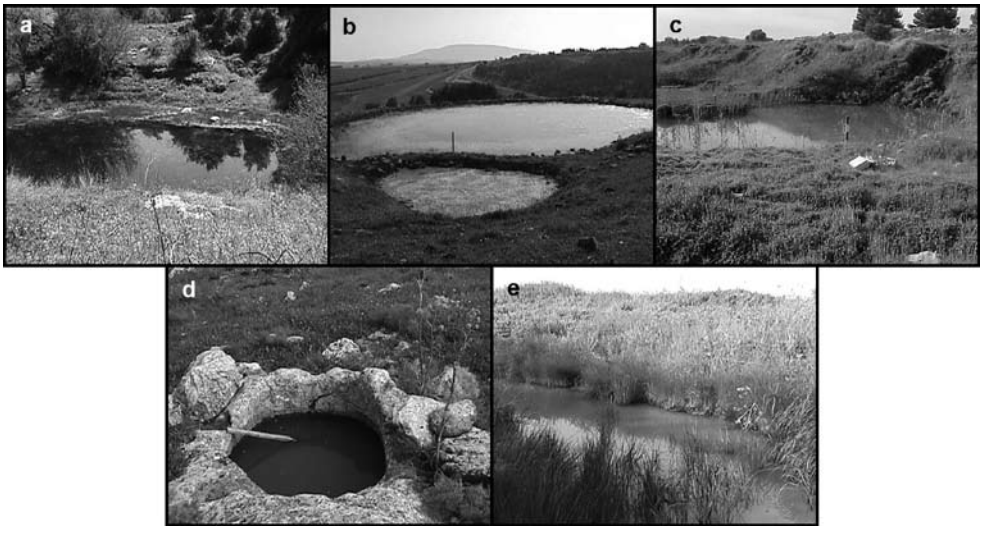

Fig. 2. The five breeding sites of *Triturus v. vittatus* examined in this study: (a) Dovev Pond, (b) Nahalit Pond, (c) Matityahu Q. Pond, (d) Amiad water holes, (e) Afeka.

drainage canal. Rainwater, draining from adjacent open fields and from the road, usually fills the pool, beginning in December and lasting until mid-March. It is ca 30 m^2 in area and ca 0.5 m deep in the center. The vegetation in the pond consists mostly of *Eleocharis palustris* and *Scirpus marifimus*. Adult *Triturus* were found in the water from December until the end of February, and larvae were observed from mid-March until mid-April, when the water dries up.

DNA analyses

Tissue samples were obtained from *Triturus* larvae and adults. As previously described by Degani and Mendelssohn (1983), adults migrate to these ponds during the mating season (at the beginning of winter) to breed. There is a large adult population at all five locations (Degani and Kaplan, 1999). Specimens (four individuals per each site) were sampled randomly from the entire area of the water body by hand net.

Total genomic DNA was extracted from tail-clipped tissues of adults or from whole tails of larvae. Extraction was carried out by the QIAamp DNA Mini Kit (Qiagen, Germany), after proteinase K digestion. RAPD PCR amplifications were carried out with 10 mer oligonucleotide primers (Mikulicek and Pialek, 2003) in a 50 µl solution, containing 10 mM Tris-HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM of each dNTP, 0.5 µM of primer, 10-500 ng gemonic DNA and 2.5 units of Taq DNA Polymerase (Promega, USA). RAPD fragments were amplified by 45 cycles, including denaturation for 30 s at 94 °C (initial denaturation for 2 min at 94 °C), primer annealing for 1 min at 35 °C and extension for 2 min at 72 °C. The RAPD amplification ended with an extension at 72 °C for 7 min. To assess the similarity between individuals, band sharing (BS) of the RAPD PCR products was calculated as: $BS = 2 \times (Nab) / (Na + Nb)$, 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).

RESULTS

Only one of the 20 primers used in the RAPD PCR was revealed to be suitable for the study of DNA variation among *T. v. vittatus* (OPA-16; 5'-AGCCAGCGAA, Mikulicek and Pialek, 2003). Other primers either failed to amplify any fragment or only amplified a few fragments, making them inappropriate for a study on variations. In PCRs with the primer OPA-16, the number of bands differed among populations from different sites. There were 12-13 identical bands within populations from Afeka Pond and the Amiad waterhole, 13-14 within the populations of Dovev Pond and Matityahu Quarry and 16-17 in the population at Nahalit Pond. Twelve bands were common to all of the newts. The newts in Nahalit, Amiad and Dovev had one band each that was not observed in any other population. Only three bands, in Nahalit, Amiad and Dovev populations, were not detected in newts from the other ponds. High genetic similarity, calculated by band sharing, was discovered among newts in the ponds at the high altitudes, which were found to have less similarity with the newts from the ponds at lower altitudes (Table 1, Figs 3, 4).

Table 1. Band sharing of newts from studied sites (see methods for details).

	Band Sharing (%)			
	Nahalit Pond	Amiad Waterhole	Dovev Pond	Afeka Pond
Matityahu Quarry	90	96	92	88
Nahalit Pond	—	80	83	80
Amiad Waterhole	—	—	88	92
Dovev Pond	—	—	—	88

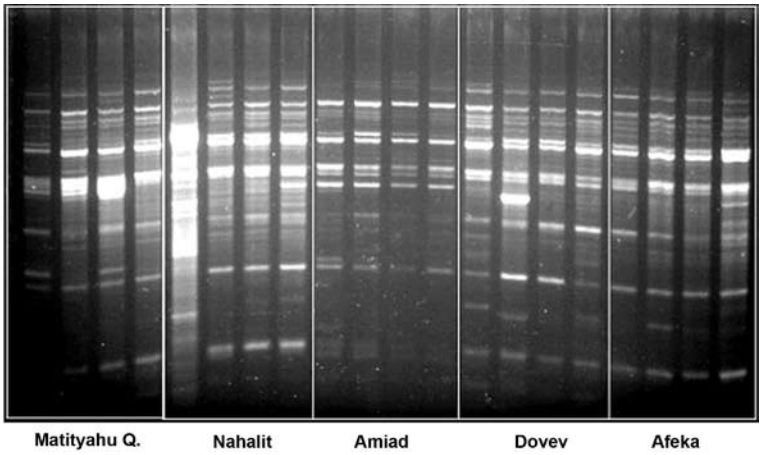

Fig. 3. RAPD PCR results with the OPA-16 primer.

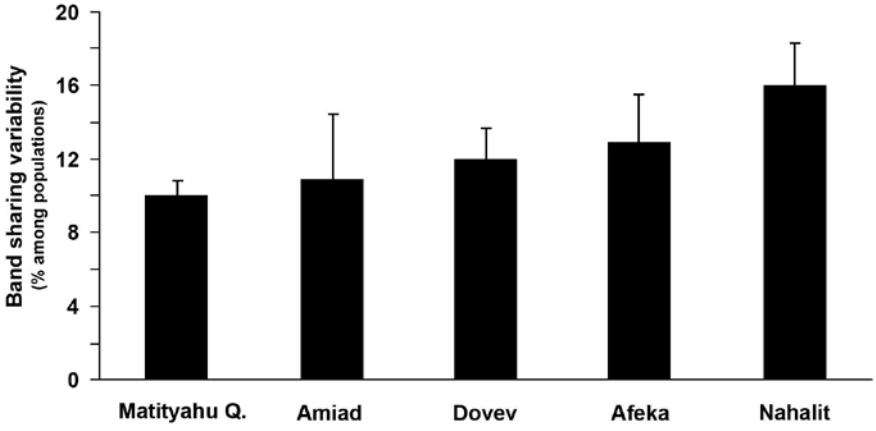

Fig. 4. Band sharing variations among populations (mean + SE).

DISCUSSION

A low genetic variability was discovered among the different *Triturus v. vittatus* populations from various altitudes and located at short geographical distances, one from the other, in Israel. Moreover, when these Israeli newt populations, which belong to the subspecies *Triturus v. vittatus*, were compared by sequence analysis to the other subspecies *T. v. ophryticus* (found in European Turkey), high genetic variations were observed (Veith et al., 2004). Mikulicek and Pialek (2003) used twenty 10-mer oligonucleotide primers on three crested newt species (*Triturus cristatus* superspecies) and found 19 of them to yield 2 to 22 bands per primer. In the present study, a very high similarity was detected and only one primer out of 20 (Mikulicek and Pialek, 2003) yielded a reasonable number of bands to show a clear variation among the populations. We believe that the explanation for this result is the very short geographical distances among the populations, together with the fact that all of the habitats were of the unpredictable type, causing the newts to adapt to the wide ecological variations. It has been previously shown that the ecological and biological conditions, suitable for *T. v. vitattus*, vary, and in unpredictable habitats, such as the rain pools in the southern limit of its distribution, conditions generally correlate with altitude (Degani and Mendelssohn, 1983; Degani, 1986a, 1996; Geffen et al., 1987; Degani and Kaplan, 1999). The effects of habitat and geographic location on genetic variation have been studied in other urodela (Steinfartz et al., 2000; Lecis and Norris, 2004; Riberon et al., 2004). Much research has been conducted on a range of habitats in widespread locations. However, very few studies have examined genetic variation among different populations at breeding sites, covering a relatively small area, as in the present study.

Triturus vittatus vittatus is a urodelan, which can be found in different freshwater bodies, such as unpredictable rain pools and springs. Water parameters, such as temperature and duration of availability in the winter ponds studied, exert a greater influence on the larvae than does the type of water body. In such ponds, the altitude of the pond influenced the species existing there (Degani and Kaplan, 1999). The results of the present study are very similar to those of Degani et al. (1999), who studied the genetic variation of salamanders from different habitats, using RAPD PCR. They discovered a very low genetic variation between two populations from semi-arid habitats (band sharing was 94%), and in contrast, a high genetic variation between populations from semi-arid and humid habitats (85-86% band sharing). Their results agree with the present study, in which ecological conditions affected genetic variation.

The foremost methods for studying genetic variation are putative proteins (Degani, 1986b) and enzymes (Veith et al., 1992), differences in DNA fingerprints (RAPD PCR; Degani et al., 1999; Mikulicek and Pialek, 2003) and sequences of conserved and variable genes (Kocher et al., 1989; Macgregor et al., 1990; Caccone et al., 1997). The methods used in this study support previous findings, demonstrating that RAPD PCR is a suitable method for examining small genetic variations among populations, whereas the utilization of cytochrome *b* sequencing may be applied when there is a greater taxonomic variation, such as between species (Steinfartz et al., 2000; Lecis and Norris, 2004; Riberon et al., 2004). Although the genetic variation among the *Triturus vittatus vittatus* populations is relatively low, one of the populations, that of Nahlit Pond, which is the most polluted pond, as mentioned previously, varied more from the other populations. In addition, the

population of Afeka Pond, which is located at a relatively low altitude and at a greater distance from the other breeding sites, differed more genetically, as compared to the other populations. Further studies are necessary to test various hypotheses that may explain the pattern discerned in the results.

REFERENCES

Beebee, T.J.C., Rowe, G., Arntzen, J.W. (1999): Identification of newts (*Triturus* sp.) and their hybrids using molecular methods. J. Zool., Lond. **249**: 43-47.

Borkin, L.J., Litvinchuk, S.N., Zuiderwijk, A. (2003): Bandmolch, *Triturus vittatus* (Gray, 1835). In: Handbuch der Reptilien und Amphibien Europas. Bd. 4/IIA: Schwanzlurche (Urodela) IIA, p. 555-605. Grossenbacher K.G., Thiesmeier B., Eds, Aula-Verlag, Wiesbaden.

Caccone, A., Milinkovitch, M.C., Sordoni, V., Powell, J.R. (1997): Mitochondrial DNA rates and biogeography in European newts (genus *Euproctus*). Syst. Biol. **46**: 126-144.

Degani, G. (1986a): Growth and behavior of six species of amphibian larvae in winter pond in Israel. Hydrobiologia **140**: 5-10.

Degani, G. (1986b): Plasma proteins and morphology of *Salamandra salamandra* in Israel. Amphibia-Reptilia **7**: 105-114.

Degani, G. (1996): The salamander at the southern limit of its distribution. Laser Pages Publ Ltd, Jerusalem, Israel.

Degani, G., Jackson, K., Dosoretz, C.G., Plotzky, Y. (1999): Molecular DNA variation in *Salamandra s. infraimmaculata* from different habitats. Isr. J. Zool. **44**: 239-246.

Degani, G., Kaplan, D. (1999): Distribution of amphibian larvae in Israeli habitats with changeable water availability. Hydrobiologia **405**: 49-56.

Degani, G., Mendelssohn, H. (1983): The habitats, distribution and life history of *Triturus vittatus vittatus* (Jenyns) in the Mount Meron Area (Upper Galilee, Israel). Br. J. Herpetol. **6**: 317-319.

Geffen, E., Gafny, S., Gasith, A. (1987): Contribution to the knowledge of the biology of the banded newt, *Triturus vittatus vittatus*, in rainpools in Israel. Isr. J. Zool. **34**: 213-223.

Gibbs, J.P. (1998): Genetic structure of redback salamander *Plethodon cinereus* populations in continuous and fragmented forests. Biol. Conserv. **86**: 77-81.

Jeffreys, A.J., Morton, D.B. (1987): DNA fingerprints of dogs and cats. Anim. Genet. **18**: 1-15.

Kocher, T.D., Thomas, W.K., Meyer, A., Paabo, S., Villablanca, F.X., Wilson, A.C. (1989): Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. U.S.A. **86**: 6196-6200.

Lecis, R., Norris, K. (2004): Population genetic diversity of the endemic Sardinian newt *Euproctus platycephalus*: implications for conservation. Biol. Conserv. **119**: 263-270.

Litvinchuk, S.N., Zuiderwijk, A., Borkin, L.J., Rosanov, J.M. (2005): Taxonomic status of *Triturus vittatus* (Amphibia: Salamandridae) in western Turkey: trunk vertebrae count, genome size and allozyme data. Amphibia-Reptilia **26**: 305-323.

Macgregor, H.C., Sessions, S.K., Arntzen, J.W. (1990): An integrative analysis of phyloge-

netic relationships among newts of the genus *Triturus* (family Salamandridae), using comparative biochemistry, cytogenetics and reproductive interactions. J. Evol. Biol. **3**: 329-373.

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

Olgun, K.V., Tok, J., Arntzen, W., Turkozan, O. (1997): The taxonomic status of the banded newt (*Triturus vittatus*) in southern Turkey. Herpetol. J. **7**: 169-171.

Parker, R.E. (1976): Introductory Statistics for Biology. The Queen's University of Belfast. Edward Arnold.

Raxworthy, C.J. (1989): Courtship, fighting and sexual dimorphism of the banded newt, *Triturus vittatus ophryticus*. Ethology **81**: 148-170.

Riberon, A., Miaud, C., Guyetant, R., Taberlet, P. (2004): Genetic variation in an endemic salamander, *Salamandra atra*, using amplified fragment length polymorphism. Mol. Phylogenet. Evol. **31**: 910-914.

Steinfartz, S., Veith, M., Tautz, D. (2000): Mitochondrial sequence analysis of *Salamandra* taxa suggests old splits of major lineages and postglacial recolonizations of central Europe from distinct source populations of *Salamandra salamandra*. Mol. Ecol. **9**: 397-410.

Veith, M., Degani, G., Seitz, A. (1992): High genetic homogeneity of *Salamandra salamandra* (L.) in Israel. Zool. Anz. **229**: 63-72.

Veith, M., Mayer, C., Samraoui, B., Donaire Barroso, D., Bogaerts, S. (2004): From Europe to Africa and vice versa: evidence for multiple intercontinental dispersal in ribbed salamanders (genus *Pleurodeles*). J. Biogeogr. **31**: 159-171.

Weisrock, D.W., Robert, M.J., Ugurtas, I.H., Larson, A., Papenfuss, T.J. (2001): Molecular phylogenetics and historical biogeography among salamandrids of the "true" salamander clade: rapid branching of numerous highly divergent lineages in *Mertensiella luschani* associated with the rise of Anatolia. Mol. Phylogenet. Evol. **18**: 434-448.

Weisrock, D.W., Papenfuss, T.J., Macey, J.R., Litvinchuk, S.N., Polymeni, R., Ugurtas, I.H., Zhao, E., Jowkar, H., Larson, A. (2006): A molecular assessment of phylogenetic relationships and lineage accumulation rates within the family Salamandridae (Amphibia, Caudata). Mol. Phylogenet. Evol. **41**: 368-383.

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

Zajc, I., Arntzen, J.W. (2000): Evolutionary relationships among European newts (genus *Triturus*) as inferred from two mtDNA fragments. Pflüg. Arch. Eur. J. Physiol. **439**: 21-22.