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The Nile Soft-shell Turtle, *Trionyx triunguis*, of Israel and Turkey: Two genetically indistinguishable populations?

(Reptilia: Testudines: Trionychidae)

Uri Shanas, Müge Gidiş, Yakup Kaska, Yael Kimalov,
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Abstract. Whereas the Nile Soft-shelled Turtle, *Trionyx triunguis*, used to be found in most of the east Mediterranean rivers, today only three major subpopulations remain: two in Turkey and one in Israel. The management of small subpopulations should rely on their genetic relatedness, and so this study examined the AFLP and *cytochrome b* genetic diversity of 58 *T. triunguis* specimens from the Alexander River in Israel and from Dalaman's Lake Kükürtlü in southwestern Turkey. The four selective primer pairs for AFLPs yielded 339 distinct loci. We found the populations to be highly polymorphic (>88%) and the level of gene diversity (H_e) relatively low (0.11). Indeed, using our methods, the two populations were found to be genetically identical ($I=1.0$). Our study further demonstrates a high identity of the mitochondrial gene *cytochrome b* DNA sequence with a Liberian (West Africa) specimen of *T. triunguis*. These results support previous preliminary genetic studies and observations which showed that this species travels around in the Mediterranean Sea. However, we suggest that the results are evidence of previous large populations and of past connections with the African populations, and that the dams on the Nile are probably preventing this gene flow today.

Key words. *Trionyx triunguis*, Mediterranean Sea, freshwater reptile, population genetics.

Introduction

Land degradation, fragmentation processes, and climate change have led to habitat loss and to a decrease in the population size of many species. This decline may have a severe outcome for species that show restricted dispersal abilities. However, species capable of long-distance dispersal can maintain a meta-population structure and thus overcome temporary declines. Among vertebrates, freshwater reptiles may be highly susceptible to the pressures of declining suitable habitats, especially in semi-arid regions, that are seriously affected by climate change and increased human exploitation. Managing such declining populations is highly desirable, but requires basic genetic information for the species.

Small isolated populations may suffer from decreased genetic variability due to a range of phenomena, including genetic drift and inbreeding depression. Consequently, such populations exhibit a loss of heterozygosity, which may have a deleterious effect on population fitness and may eventually lead to population extinction (reviewed in REED & FRANKHAM 2003, ALLENDORF & LUIKART 2007). Gene diversity was suggested as a target for conservation by the International Union for Conservation of Nature (IUCN) (MCNEELY et al. 1990), and since then has gained popularity, and new tools have been developed and applied (SCHWARTZ et al. 2007, DUDGEON et al. 2012). Indeed, field experiments in the seagrass

Zostera marina have indicated that ecosystem resilience may be strengthened by increasing genetic diversity (REUSCH et al. 2005). Understanding the genetic structure of different populations within the same species can provide a useful tool for management. For example, genetic data for the New Zealand Tuatara (*Sphenodon punctatus*) support the current management of conserving three different lineages despite its being described as a single species (HAY et al. 2009).

The Nile Soft-shell Turtle (*Trionyx triunguis* (Forskål, 1775)) is among the largest freshwater turtles (up to 120 cm) that inhabit shallow water bodies, including rivers and marshes. The species has a wide ranging distribution in Africa, especially in the rivers of West Africa and in the Nile River system (GIDIŞ et al. 2010). It is assumed that the species extended its distribution into the east Mediterranean Sea through the Nile River. The current situation of this species in the Mediterranean is unclear. KASPAREK & KINZELBACH (1991) described sites that were previously unknown, but they warned against the increased threats that *T. triunguis* faced in this region and they assessed the population size to be less than a thousand. In Israel, for example, *T. triunguis* used to be abundant 60 years ago (according to unofficial censuses: ARBEL 1984) in all the coastal rivers of the east Mediterranean, but now only a single river, the Alexander River, holds a population of over 100 individuals while in other rivers only a few individuals can be sighted. Until recently *T. triunguis* in the Mediterranean was listed in the Red Book by the IUCN (European Reptile and Amphibian Specialist Group, 1996, category CR C2A), but has been taken off the list (IUCN 2010) due to the unproven decline in its population.

T. triunguis populations in the east Mediterranean Sea remain in three major disconnected populations: the Dalaman area and Seyhan River in Turkey and the Alexander River in Israel (KASPAREK 1999, 2001, KASPAREK & KINZELBACH 1991). The species is considered extinct in Egypt. In Lebanon and Syria, there are only a few recent records and it is uncertain whether reproduction occurs in these countries (KASPAREK, unpubl.). Furthermore, the current populations of the turtle are under constant threat from commensal predators (Red Fox, and the Egyptian Mongoose, *Herpestes ichneumon*), and from the deteriorating water quality of their habitat.

The patchy distribution of *T. triunguis* in the east Mediterranean and the declining number of populations of the species in this region may have led to a decrease in its genetic variability. Recent sequencing analyses of two mitochondrial and five nuclear markers indicated a remarkably low level of genetic diversity between and within *T. triunguis* populations sampled across the species, ranging from Cameroon in West Africa through the Nile River to Turkey (GIDIŞ et al. 2010). However, the discrimination of weakly differentiated populations should be based on a large number of loci, to overcome the lack of statistical power. One technique that generates a large number of genetic markers with consistently high assignment success (CAMPBELL et al. 2003) is that of amplified fragment length polymorphism (AFLP: VOS et al. 1995). This study examined the AFLP and *cytochrome b* genetic diversity of two east Mediterranean populations of *T. triunguis* from Israel and Turkey in order to elucidate the diversity within and between the two populations.

Material and methods

Sampled populations. We trapped 26 *T. triunguis* individuals from the Alexander River in Israel (32°23'N, 34°53'E) and 10 individuals from Lake (Sulphurous) Kükürtlü in Dalaman, Turkey (36°41'N, 28°47'E). The Turkish population was supplemented with an additional 22 individuals that were collected in the same location (Lake Kükürtlü) and had been stored in alcohol, making

a total of 58 *T. triunguis* individuals. The turtles were trapped with a 60 x 80 cm net (mesh size 3 x 3 cm). We sampled blood from the specimens caught in Israel by inserting a needle (19-22G) connected to a 5 ml syringe into the caudal vein. The extracted blood was transferred to a 10 ml Lithium-Heparin tube (Estar Ltd.) and was stored in a cold container until centrifuged a few hours later. The red blood cell layer was then stored under -18°C until analysis. We sampled the Turkish population by taking either a cartilage sample or a muscle sample from the additional specimens provided by GIDIŞ & KASKA (2004).

Genetic analysis. Turtle DNA was extracted from blood or from the soft-shell inner tissue (cartilage or muscle) of preserved specimens, using DNeasy Blood and Tissue Kit (Qiagen), with minimal modifications. The genetic study included AFLP fingerprint analysis and sequence analysis of 500 bp of the mitochondrial gene *cytochrome b*.

The AFLP method was essentially carried out as described by VOS et al. (1995). High-quality genomic DNA (~200 ng) was digested with a pair of restriction enzymes (*EcoRI/MseI*) at 37°C for four hours, and then ligated to double-stranded *EcoRI* (E-) and *MseI* (M-) adaptors. The resulting fragments were pre-amplified with non-selective primers, where the ligated adaptors served as target sites for primer annealing. Four selective primer combinations were used for AFLP amplification: E-ACA/M-CAC; E-ACG/M-CTT; E-ACT/M-CAA; and E-ACC/M-CTG (with E- and M- representing the restriction site and its ligated adaptor sequence). The selective *EcoRI* (E-) primers were labeled with florescent dye (6-Fam, Ned, Vic, and Pet).

PCR reactions were carried out in a total volume of 13 microliters. PCR amplification cycles started at an annealing temperature of 65°C, after which the annealing temperature was lowered by 0.7°C per cycle for 12 cycles (with a touch-down phase of 13 cycles), followed by 23 cycles at an annealing temperature of 56°C. The amplification products were visualized under a Fluorescence-Reader (Applied Biosystems). Allele identification and genotyping were determined directly from the chromatographs using Peak Scanner software (Applied Biosystems).

Cytochrome b sequencing: A fragment of the mitochondrial gene was amplified by PCR, with the primers' design following the sequences found in GenBank (accession numbers: AY259564; primers: F: 5'-ACCCGRGAYGTACAATACGG-3'; R: 5'-GGATGGAGGCTRYTGTCCRAT-3') in a total volume of 13 microliters at an annealing temperature of 50°C. The single PCR products were purified (QIAquick columns – Qiagen) and sent for direct sequencing.

Genetic data analysis. Sequence analysis: All chromatographs were checked manually for their reliability. Sequences were aligned using the program ClustalX 1.81 (THOMPSON et al. 1997) and were compared with the published sequences of *T. triunguis* collected in Liberia, Africa (AY259564; ENGSTROM et al. 2004). As an outgroup, the published sequence of the Florida Soft-shell Turtle, *Apalone ferox* (AY259555; ENGSTROM et al. 2004) was used.

AFLP analysis: Amplification products were scored as discrete character states (present/absent) and transformed into band frequencies. Diversity values were based on phenotype frequency, and phenotypes were defined by the band patterns produced by individual primer pairs. Data were analyzed by GenAlEx (PEAKALL & SMOUSE 2006). This program considers AFLP bands as diploid-dominant markers in which the estimated allele frequencies are based on the square root of the frequency of the null (recessive) genotype.

Bayesian partitioning clustering was applied using the program STRUCTURE 2.3.3 (PRITCHARD et al. 2000, FALUSH et al. 2007). Combining the data there were collection sites for 2 samples (a putative number of populations - K). A Structure run was applied, with 100,000 burn-in iterations and 50,000/500,000 MCMC reps, with Ks ranging from 1 to 3 (ten replicates for each K).

The inference of the probable number of clusters was extracted by the log likelihood for each K, $L_n P(D) = L(K)$, using the program Structure Harvester (EARL & VON HOLDT 2012).

Table 1. Genetic diversity parameters revealed by AFLP analysis.

Population	Israel	Turkey
N	26	32
No. of Different Bands	299	304
No. of Bands Freq. $\geq 5\%$	241	254
No. of Private Bands	35	40
Percentage of Polymorphic Loci	88.2%	89.7%
Mean H_e (unbiased)	0.105	0.126
SE of Mean H_e	0.005	0.005
I (Shannon's Information Index)	0.197	0.226
SE of I	0.007	0.008

Results

The four selective primer pairs yielded 339 distinct loci. The level of polymorphism was relatively high (>88%) (Table 1); hence, the level of gene diversity (H_e) was relatively low (0.11). The two populations were genetically identical ($I=1.0$, Nei Unbiased Genetic Identity). Analysis of molecular variance (AMOVA) indicated that there was no difference in variance between the populations and that all the molecular variance (100%) was found within the populations. Nonetheless, the Turkish population exhibited a slightly higher genetic variability level in all tested parameters, namely, the number of different bands, the number of private bands, the percentage of polymorphic loci, the level of gene diversity, and Shannon's index (see Table 1).

Sequence analysis of *cytochrome b* gene showed high identity between all tested sequences (Israel, Turkey, and Liberia-Africa). There was no evidence for any differentiation between the turtles found in Liberia (Africa), Israel, and Turkey. There was one common consensus haplotype, and the identity between all tested and compared sequences exceeded 99%.

Structure analysis of the two localities showed no difference in genetic composition (Fig. 1). The lowest log probability of data $L(K)$, i.e. the probable number of clusters, was found for $K=1$.

Discussion

The current results, which are based on relatively large samples and on multi-locus comparison, support earlier observations and genetic results (GIDIŞ et al. 2010, GÜÇLÜ et al. 2009) that the local *T. triunguis* in the east Mediterranean Sea should be regarded as a single population throughout its range. Apparently the >700 km of highly saline sea that separates Dalaman, Turkey, from Israel is not a barrier for this species. Indeed, previous observations have found live specimens in the east Mediterranean Sea (KASPAREK 2001), which may indicate their ability to cross large bodies of marine water.

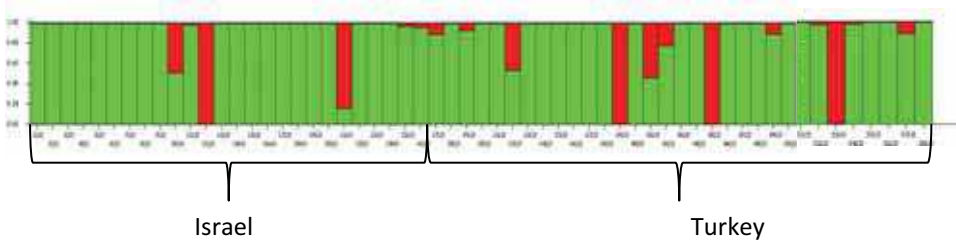


Fig. 1. The results of a STRUCTURE Bayesian cluster analysis assuming two populations (Israel, Turkey) of *Trionyx triunguis*. The colours (grey tones) indicate different clusters (i.e., admixture groups). Each bar (dark or light) represents an individual turtle (X axis), while bar height (Y axis) indicates the probability that each individual belongs to the respective admixture group.

In the study by GÜÇLÜ et al. (2009), based on mitochondrial DNA, there is the suggestion of isolation between African and Turkish populations. Reptiles in general and turtles in particular are reported to have a relatively slower rate of evolutionary change compared with other placental vertebrates of the same size (WILSON et al. 1975, AVISE et al. 1992). Therefore, it is possible that the use of mitochondrial data is not sensitive enough to track population differences. For example, FRITZ et al. (2007) found in the Balkan Terrapin, *Mauremys rivulata*, nuclear but not mitochondrial differentiation between populations in Turkey and the Jordan River basin. GÜÇLÜ et al. (2009) suggest the addition of nuclear data to confirm their finding of a genetic interconnection between Mediterranean populations. In our study, we chose to use the AFLP method since it is one of the most sensitive methods for tracking genetic differences between populations (CAMPBELL et al. 2003). We can therefore assume that if Israeli and Turkish *T. triunguis* represent genetically distinct lineages, this would probably have been tracked by our method. By contrast, we found a high level of polymorphism (>85%) in the genetic material of both locations.

Our study further demonstrates a high similarity in the mitochondrial gene *cytochrome b* DNA sequence with a Liberian specimen of *T. triunguis*. This specimen is from a site even further west than the Cameroon turtles used in GIDIŞ' study (2010). Apparently, similar to the Cameroon population, the Liberian population used to be connected with the Mediterranean population through the Niger-Congo-Nile Rivers, as suggested by GIDIŞ et al. (2010). One may think that based on the previous (GIDIŞ et al. 2010) and the current studies, the decision to remove this species from the IUCN red list (IUCN 2010) appears to be sound. However, the Mediterranean population, though not unique according to these studies, is under increasing threat from direct exploitation, habitat degradation, and predation by increasing numbers of mesopredators. A flow of African individuals that apparently have some genetic differences from the Mediterranean one (GÜÇLÜ et al. 2009) and an ongoing gene flow among populations could somehow inhibit the severe outcomes of these processes, but apparently only the latter process is taking place. This gene flow among locations in the east Mediterranean basin will enable a meta-population structure, and if in the future any of these populations collapses an assisted reintroduction programme could be considered,



Fig. 2. Nile Soft-shelled Turtle, *Trionyx triunguis*, at Nahar Aleander, Israel (photograph: M. KASPAREK).

using individuals from one of the main sites of occurrence (KASPAREK & KINZELBACH 1991) as a source.

The current results showing genetically indistinguishable populations across the range of this turtle are probably an outcome of the past connections of the Mediterranean with the African turtles through the Nile River. Unfortunately, this connection has been interrupted by the construction of the Aswan Dam, first in the early 20th century and later with the construction of the new dam in the 1970s. Indeed, *T. triunguis* has disappeared from the Nile delta and from Egypt entirely (NADA 2002). In order to enable future gene flow among the *T. triunguis* sub-populations, it would be necessary to reintroduce turtles to the lower Nile River and to actively exchange individuals across the Nile dams.

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