

**Extremely low genetic diversity in the endangered striped nerite,
Theodoxus transversalis (Mollusca, Gastropoda, Neritidae)
– a result of ancestral or recent effects?**

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Abstract. The striped nerite (*Theodoxus transversalis*), once a widespread freshwater snail, is of high conservation priority as the majority of its populations have become extinct during the past few decades. We have performed a preliminary study to calculate intraspecific variability of the *T. transversalis* using two different DNA markers, the mitochondrial cytochrome c oxidase subunit I (COI) and the intron of the nuclear-coded ATP synthase subunit α (ATPS α). Surprisingly, the intraspecific variability is very low; we have found only three closely related haplotypes of each marker. Considering that the sampling sites represent well the whole present range of the species, this phenomenon seems to be the consequence of a historical bottleneck. In our view, the whole Holocene range has been colonized by descendants of a bottlenecked population. Low genetic diversity, however, was not an obstacle for this species to obtain a relatively wide range and abundance until the recent past. Comparison with other *Theodoxus* species of similar sized historical ranges raises the idea that possibly reduced genetic diversity accounts for the species' high sensitivity to water quality and habitat alteration. Based on these data, the species represents a single management unit for conservation, i.e. each viable population shall play an equal role in the preservation of the species in the future. To prevent the extinction of *Theodoxus transversalis*, high priority should be directed to the maintenance of suitable habitat conditions as well as monitoring the conservation status of persistent populations.

Key words: bottleneck, demographic history, intraspecific molecular diversity, mismatch distribution, mitochondrial COI, nuclear-coded ATPS α , *Theodoxus transversalis*.

Introduction

The "central dogma" of conservation genetics is that genetic variability is beneficial, hence its preservation is among the principal aims of conservation biology (Pertoldi et al. 2007). This should also be true for intraspecific genetic diversity, which is known to play a very important role in the long-term survival and adaptability of a species. Thus its knowledge provides essential information for the systematic conservation planning of threatened species. On the other hand, spatial structure and distribution of this diversity bears the marks of past events, thus it also provides usable information for a better understanding of the species' biogeographical history as well (Hickerson et al. 2010).

Theodoxus transversalis C. Pfeiffer, 1828 is a freshwater nerite species of mainly Danubian distribution. Once widespread within the Danube drainage, it was a characteristic species of the fluvial assemblages of the Danube and its larger

tributaries. Due to decreasing water quality and habitat alterations, there was severe decline in the number of populations during the past few decades, thus nowadays the known sites of this species are only around ten (Hubenov 2007; ICPDR 2002, 2008; Karaman 2005; Sirbu & Benedek 2005; Tittizer & Banning 2000; Varga 2008; Zettler 2008; Živić et al. 2000). Due to this rapid decline and present rarity, *T. transversalis* is listed by the Annexes II and IV of the European Habitat Directive and categorized as Endangered by IUCN (Sólymos & Fehér 2010).

There have been molecular studies focusing on other *Theodoxus* species (Bunje 2005, 2007, Fehér et al. 2009), but so far *T. transversalis* was only superficially studied (Bunje & Lindberg 2007). All that was revealed is that this species is the sole member of the most basal clade of the genus, split from the rest of the genus during the Early Oligocene (ca. 30 My before present) and considered therefore as a surviving relict of the genus' earliest diversification (Bunje & Lindberg 2007).

In the framework of a larger project, which is dealing with the freshwater mollusc fauna of the Balkans and the Carpathian Basin, we have studied several fluvial biotopes within the Danube drainage and in the Balkans. During this project, a significant proportion of the remaining *T. transversalis* populations was sampled. As these samples represent different hydrological subunits of the former range of the species (including the Axios river, which does not belong to the Danube drainage), they provided a good opportunity to study the species' intraspecific diversity at the large scale and to try to answer the following questions: (i) How large is the genetic variability, preserved in the fragmented surviving populations? (ii) If there is a hot spot area of intraspecific variability, where is it? (iii) Are there distinct intraspecific clades, which could be considered distinct conservation entities? (iv) What kind of conclusions can we draw about the biogeographical past of this species? (v) And finally, on which areas/populations should species conservation actions focus?

Material and methods

Fieldwork was carried out from 2006 to 2009. For conservation reasons, only a few (2–4) living specimens were collected by location. Sampling sites are listed in Table 1 and are shown on the map of Fig. 1. The specimens were hand collected in the field and put into 96% ethanol. This is a general method for fixing and preserving freshwater molluscs for DNA study, causing minimal pain and inflicting immediate death of the animals. Samples have been deposited in the Mollusca Collection of the Hungarian Natural History Museum.

We have studied the DNA sequences of mitochondrial cytochrome c oxidase subunit I (COI) [15 specimens] and the intron of the nuclear-coded ATP syntase subunit α (ATPS α) [10 specimens]. As three localities along the Bódva river can practically be considered as one population, six specimens were selected from the Bódva samples to study the intra-population variability. DNA extraction, COI amplification and sequencing have been carried out according to Fehér et al. (2009), ATPS α amplification and sequencing were done according to Jarman et al. (2002).

Sequences were aligned by ClustalW as implemented in MEGA version 5.05 (Tamura et al. 2011). For reasons of comparability, COI haplotypes were aligned to the available 600 bp long *Theodoxus* sequences (Bunje 2007, Fehér et al. 2009) and cut accordingly. ATPS α sequences were 479 bp long (including primers and some flanking exon regions – 93 bp on the 5'-end and 48 bp on the 3'-end). Identical sequences were then collapsed into haplotypes. Haplotype frequencies are listed in Tables 1–2 and sequences of individual haplotypes were deposited in the

GenBank (HM171586–HM171593, JQ678844–JQ678846).

Haplotype networks were constructed using the statistical parsimony procedure as implemented in TCS version 1.21 (Clement et al. 2000). Arlequin version 3.11 (Excoffier et al. 2005) was used to perform further analyses on the COI data; nucleotide diversity (π) and mean pairwise genetic distances were calculated as well as Tajima's D test of selective neutrality and mismatch distribution analysis were performed. We tested the validity of the sudden expansion model for our observed data, derived from the mismatch distribution analysis, using the SSD (Sum of Square Deviation) statistics. Harpending's Raggedness index (Ri) was also calculated to test the unimodality of observed data, derived from the mismatch distribution analysis.

For comparison, the same analyses were performed with two sets of COI sequences, (i) those of *T. danubialis* from the Danube drainage, which is a species of a comparable sized range (Table 2) and (ii) those of northern European *T. fluviatilis* populations (excluding the so-called „*T. velox* clade”), which are of well-known demographic history (Bunje 2005).

Results

Very low intraspecific variability was found among the studied *T. transversalis* specimens. Fifteen specimens from eight localities were found to belong to a total of three COI haplotypes. The most frequent one (TT1) occurred in all but one localities. The other two haplotypes were found in one locality each; TT2 in the Bódva river and TT8 in the Zlatna Panega river (Table 1). The frequent TT1 haplotype differs from TT2 and TT8 in one and two base pairs respectively (Fig. 1). All of the three mutations occurred at the 3rd codon position and none of them resulted in amino-acid mutation.

In the ATPS α marker, there were only two variable sites and the ten specimens belonged to three haplotypes altogether. Both sTT2 and sTT3 occur in the Zlatna Panega population, the latter one also in the Axios river, whereas sTT1 haplotype was found in the Bódva, Hernád and Tisza populations (Fig. 1).

Genetic diversity indices for this *T. transversalis* COI sequences were lower than not only those of *T. danubialis*, but even lower than the northern European *T. fluviatilis* samples (Table 3). Mismatch analysis has shown that *T. transversalis* pairwise differences form an unimodal distribution with a mode near zero. The same was found for northern European *T. fluviatilis*, except that the mode of its mismatch distribution is somewhat higher. In contrast, for *T. danubialis*, we have

Table 1. List of *Theodoxus transversalis* sampling locations. Country, river, nearest settlement and geographical position are provided for each sampling site, as well as GenBank accession numbers and frequency of each haplotype sampled at a location.

code	Locality (country, river, nearest settlement)	Geographical position	COI			ATPS α		
			Haplotype	GenBank Number	Frequency	Haplotype	GenBank Number	Frequency
B1	Hungary, Bódva, Szendrő	N48°22.475' E20°43.647'	TT1	HM171591	2	-	-	-
			TT2	HM171593	2	-	-	-
B2	Hungary, Bódva, Edelény	N48°17.740' E20°44.408'	TT1	HM171591	1	sTT1	JQ678845	1
B3	Hungary, Bódva, Szendrőlád	N48°19.956' E20°43.836'	TT1	HM171591	1	sTT1	JQ678845	1
R	Hungary, Rába, Rábahídvég	N47°02.734' E16°40.062'	TT1	HM171591	3	-	-	-
T	Hungary, Tisza, Tiszabecs	N48°05.851' E22°49.696'	TT1	HM171591	1	sTT1	JQ678845	1
H	Hungary, Hernád, Gesztely	N48°06.478' E20°57.664'	TT1	HM171591	1	sTT1	JQ678845	2
A	Greece, Axios, Polikastro	N40°59.246' E22°33.523'	TT1	HM171591	3	sTT3	JQ678844	2
Z	Bulgaria, Zlatna Panega, Lukovit	N43°12.312' E24°09.830'	TT8	HM171592	1	sTT2	JQ678846	1
						sTT3	JQ678844	2

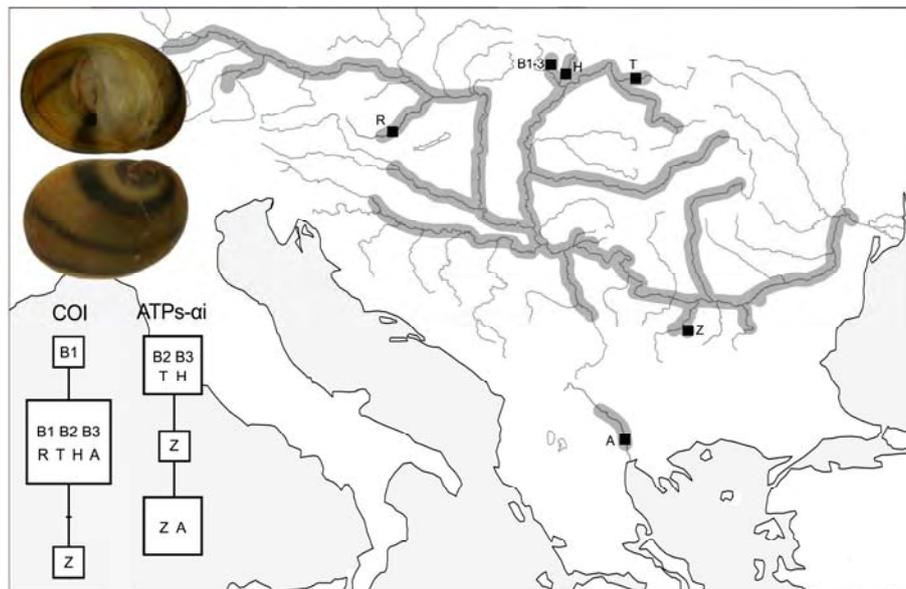


Figure 1. Haplotype networks of the studied *Theodoxus transversalis* material and location of the sampling sites. Populations are abbreviated as follows: B1-3, Bódva river; H, Hernád river; T, Tisza river; R, Rába river; Z, Zlatna Panega river; A, Axios [= Vardar] river (see also Table 1). Shaded area indicates the former geographic range of *Theodoxus transversalis* based on collection materials and literature records (Hubenov 2007, Glöer 2002, Sirbu & Benedek 2005, Karaman 2005, Soós 1906, 1943, Zettler 2008, Živić et al. 2000).

found a bimodal mismatch distribution, where the first mode is around 2-3, the second one is at 12 base pair differences. SSD values were not significant (as derived from p-values) and hence we

cannot reject the sudden expansion model in favour of equilibrium for any of the three species. All the three Tajima's D values were negative, and although none of the Tajima's D statistics were

Table 2. Frequency table of yet unpublished *Theodoxus danubialis* COI haplotypes used as comparative material in this study. Country, river, nearest settlement and geographical position are provided for each sampling site, as well as GenBank accession numbers and frequency of each haplotype sampled at a locality.

Locality	GenBank No.	AY771281	AY771313	GQ365727	GQ365726	GQ365720	HM171586	HM171587	HM171588	HM171589	HM171590
	Haplotype code	TDI (=D6)	TDKZ (=D14)	TDPB	TDD1	TDI3	Tdan06 (=TDSM)	Tdan10 (=TDS)	Tdan15 (=TDH)	Tdan16 (=TDR)	Tdan18 (=TDL)
Bosnia–Hercegovina, Fojnica, Visoko (43° 59.213' N 18° 10.992' E)					1						
Bosnia–Hercegovina, Sana, Sanski Most (44° 44.537' N 16° 40.983' E)							1				
Bosnia–Hercegovina, Una, B. Krupa (44° 54.155' N 16° 09.947' E)		1									
Bosnia–Hercegovina, Una, Rudice (45° 00.903' N 16° 21.676' E)		1									
Bulgaria, Danube, Novo Selo (44° 09.863' N 22° 47.072' E)		1									
Bulgaria, Danube, Somovit (43° 41.388' N 24° 46.224' E)		1									
Bulgaria, Danube, Vardim (43° 36.760' N 25° 28.766' E)		1									
Bulgaria, Zlatna Panega, Lukovit (43° 12.312' N 24° 09.830' E)											1
Bulgaria, Zlatna Panega, Rumjancevo (43° 07.920' N 24° 08.793' E)										1	
Croatia, Kupa, Sisak (45° 29.147' N 16° 22.244' E)								1			
Croatia, Una, H. Kostajnica (45° 13.323' N 16° 30.677' E)		1									
Hungary, Dráva, Órtilos (46° 17.890' N 16° 53.204' E)		1									
Serbia, Beli Timok, Vratarnica (43° 47.187' N 22° 18.456' E)						1					
Slovenia, Sava, Hrastnik (46° 06.690' N 15° 06.918' E)									1		
Slovenia, Sava, Krško (45° 57.514' N 15° 29.164' E)			1								

Table 3. Genetic variability indices, Tajima’s D, SSD and Harpending’s Raggedness index statistics for the COI sequences of three *Theodoxus* species. *T. fluviatilis* results were recalculated from the raw data of Bunje (2005), *T. danubialis* calculations are based on the data of Bunje 2007, Fehér et al. 2009 and unpublished samples (see Table 2).

	<i>T. transversalis</i>	<i>T. danubialis</i> (Danube drainage)	<i>T. fluviatilis</i> (Northern Europe)
No. of locations	8	37	37
No. of specimens	15	111	160
No. of haplotypes	3	24	15
Nucleotide diversity (π) [SD]	0.0009 [0.0009]	0.0087 [0.0047]	0.0018 [0.0013]
Mean pairwise differences [SD]	0.51 [0.46]	5.21 [2.54]	1.05 [0.70]
Tajima’s D	-1.317	-0.578	-1.380
	p=0.086	p= 0.329	p=0.064
SSD statistic	0.001	0.029	0.002
	p=0.64	p=0.34	p=0.27
Harpending’s Raggedness Index	0.218	0.026	0.057
	p=0.61	p=0.49	p=0.34

significant at the 5% level, for both for *T. transversalis* and *T. fluviatilis* those were below 10% (Table 3).

Discussion

Intraspecific diversity and demographic history

Due to conservation considerations we sampled only a few individuals in each location. We were

aware of the fact that low sample size might weaken the statistical support for the results, but regarding the broad representative geographical sampling we believe that our data approximate the whole intraspecific diversity and, at least, allows us to draw preliminary conclusions.

Regarding diversity at the intraspecific level, we hypothesized that contemporary range fragmentation and population extinctions resulted a loss of the former intraspecific diversity, i.e. the

extinction of several historical haplotypes and some of the historical lineages, as have been found e.g. by Johnson et al. (2007) for prairie-chicken, Weber et al. (2004) for Guadalupe fur seal, La Haye et al. (2011) for common hamster, or by Tracy & Jamieson (2011) for New Zealand mohua. Therefore, we expected that surviving *T. transversalis* populations would host some genetically divergent lineages. We also expected that the COI marker will show sufficient intraspecific variability to reveal such patterns, because it was successfully used for similar purposes in *T. transversalis* congeners (Bunje 2005, 2007). Though mitochondrial markers are widely used at the intraspecific level, they reflect the history of the mitochondrion which is not always concordant with the history of the species (Funk & Omland 2003). In order to confirm the mitochondrial results and to get more reliable inference for the species' history, we used a nuclear marker as well (ATPSa), the variability of which is comparable to mitochondrial genes (Jarman et al. 2002).

Contrary to our expectations, we found hardly any COI and ATPSa variability in *T. transversalis*; both DNA marker revealed only one lineage and only three, closely related haplotypes. Low variability of these markers in *T. transversalis* might question the applicability of the statistics and population genetic methods used. It is obvious that we need a more variable marker to make finer demographic inference. In comparison with related species, however, these results are indicative.

The unimodal pattern of pairwise genetic differences, as well as the genealogy suggest a recent demographic expansion (Rogers & Harpending 1992, Slatkin & Hudson 1991). Negative Tajima's D value – though not significant at the 5 % level

($p = 0.086$, Table 2) – can also be an indication of any kind of non-neutral evolutionary process, among others a population expansion. Neither SSD ($p = 0.64$) or Ri ($p = 0.61$) tests contradict a possible demographic expansion, as non-significant results indicate that the studied population is far from equilibrium. Similar features were found for the northern European *T. fluviatilis* populations. Those haplotypes form a typical star-like genealogy; with a widely distributed, frequent internal haplotype and several local haplotypes 1–2 bp distant to that (Bunje 2005). These populations also show low –but somewhat higher– genetic variability, a unimodal mismatch distribution and similar probability ($p = 0.064$) in the Tajima's D statistic (Table 2, Fig. 2).

The demographic history of *T. fluviatilis* in northern Europe was inferred by corroborating molecular, paleontological and paleogeographical evidence (Bunje 2005). As the most frequent northern European *T. fluviatilis* haplotype can also be detected in northern Italy, this area is assumed to be the source of the colonization event. Haplotypes in the tips of the star-like genealogy can be found within narrow ranges in northern Europe and they are supposed to evolve *in situ*. In view of the fact that this colonization happened after the glacial retreat in the Holocene, it is noteworthy that the northern European *T. fluviatilis* populations' genetic diversity is somewhat higher than that of *T. transversalis* and the mode of their pairwise differences is farther from zero. It should be noted that there is another group of *T. fluviatilis* haplotypes in northern Europe (the so called „*T. velox* clade“), but that was left out of the analyses, because of its different origin (Pontic) and the unknown date of its invasion (Bunje 2005).

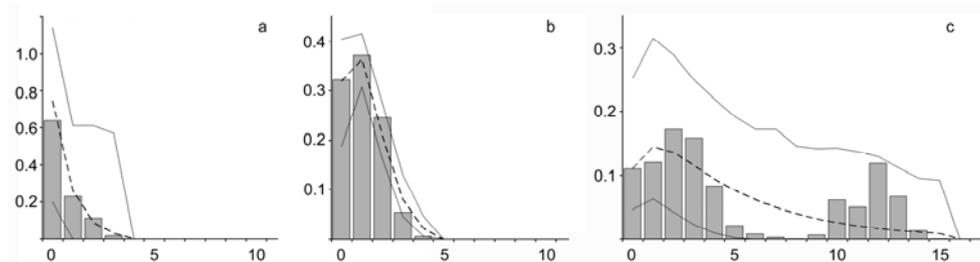


Figure 2. Pairwise mismatch distributions for *Theodoxus* COI sequences based on (a) *T. transversalis*, (b) *T. fluviatilis* (northern Europe), and (c) *T. danubialis* (Danube drainage). Thick solid lines represent the observed frequency of pairwise differences, dashed lines represent the expected distribution under the sudden expansion model and thin lines represent the lower and upper bounds of the 95% confidence interval. *T. fluviatilis* results were recalculated and redrawn from the raw data of Bunje (2005), *T. danubialis* calculations are based on the data of Bunje (2007), Fehér et al. (2009) and formerly unpublished samples (listed in Table 2).

As *T. transversalis* sampling sites are distributed range-wide, we might assume that the pattern found represents the species' genetic variability before contemporary population extinctions. Of course, it is impossible to exclude whether extinct *T. transversalis* populations carried other intraspecific lineages, but it seems feasible that present-day extinctions affected only less frequent tip haplotypes of a former star-like genealogy.

A reasonable explanation for this phenomenon is a historical bottleneck, which was followed by rapid demographical and range expansion, and accordingly, the whole Holocene range of this species was inhabited by the descendants of the same population. Though it is generally risky to estimate demographic events on the basis of genetic distance alone, in this case *T. fluviatilis* provides a usable calibration point. Therefore, if we suppose that COI gene's mutation rate is not substantially different in the two *Theodoxus* species, we might presume that demographic expansion of *T. transversalis* happened later than the colonization of northern Europe by *T. fluviatilis*, i.e. it should have happened during the Holocene epoch.

There are increasing number of case studies when a species is found to be of low genetic variability (Bisconti et al. 2011; Chan et al. 2011; Duran et al. 2004; Weber 2004; Wörheide 2005), and there are proven cases when (following a historical bottleneck) the low diversity of a species survived for a long time (Johnson et al. 2009). Weber (2004), therefore, argues that intraspecific variability in itself may give a misleading snapshot of the genetic health of the species and be of limited relevance in assessing its likelihood of persistence. In spite of this it is still widely assumed that intraspecific genetic diversity plays a very important role in the long-term survival and adaptability of a species (Frankham 2005).

Its abundance and relatively wide range until the middle of the 20th century indicate that despite reduced genetic diversity, *Theodoxus transversalis* was seemingly in a good overall condition. On the other hand, this species seems to be more susceptible to decreasing water quality and habitat alterations than the related *Theodoxus danubialis*, which has a similar range and habitat preference. There is a striking difference between the two related species in their intraspecific COI diversity and in this preliminary stage of the study it seems to be a possible explanation for the higher vulnerability of *T. transversalis*.

Faunistical notes and conservation implications

Having investigated several fluvial biotopes throughout the former *T. transversalis* range, we can ascertain that *T. transversalis* has become a very rare species today. The existence of six populations has been confirmed (three locations at the Bódva River probably belong to the same metapopulation). Even if we suppose that other occurrences, reported during the last decade, still exist (Alz river in Bayern (Zettler 2008), Jelenački stream in Serbia (Živić et al. 2000)), some locations at the Lower-Danube (ICPDR 2002, 2008) and the Južna Morava river (Karaman 2005)), the number of the remnant populations is alarmingly low (see also Csányi et al. 2005, Csányi & Paunović 2006). Beside its extra Danubian occurrence, the population in the Axios River worth some faunistical notes. First, this is the first record of this species in the Greek fauna (Bank 2011). Second, this finding reinforces Pavlović's data (see: Tomić 1959), who had already reported *T. transversalis* a century ago from the same river [Axios = Vardar] near Skopje in Macedonia. Finally, it supports the sub-recent occurrence of this species in the nearby Lake Doiran (Fischer et al. 2009). The Axios river locality lies within the territory that was occupied by several long lived lakes during the Neogene. These lakes harboured a rich fauna of *Theodoxus*, as is apparent from fossil findings (Bandel 2001; Harzhauser & Mandić 2008).

Some of the persistent populations are in worrisome conditions. Comparing our observations to literature data (Richnovszky & Pintér 1979), the population density was alarmingly low both in the Tisza and the Hernád rivers. This is the consequence of a severe decline, rather than a periodical population, caused by the siltation of the substrate in the riverbed (Varga 2008). In the Hernád river, population decline has reached such an extent that its extinction seems unavoidable in the near future. The same is true for the very small and vulnerable population of the Jelenački stream, which is threatened mainly by the untreated wastewater discharge of the town Irig (Živić, Belgrade, pers. comm. 2010). On the other hand, there are at least four populations – those of the Rába and the Bódva rivers in Hungary, the Zlatna Panega river in Bulgaria and the Axios river in Greece –, which seem to be stable and viable.

From the point of view of practical conservation planning, it has no particular importance that low diversity is the consequence of a Holocene bottleneck rather than present-day population ex-

tinctions. Practically, each viable population shares the same conservation concern and each of them shall play equal roles in the preservation of the species in the future. In order to preserve *T. transversalis*, it would be necessary to confirm literature records, to search for further populations in unsurveyed locations, to monitor the conservation status of each persistent population and to strictly protect those habitats where stable and viable populations still occur.

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