

# An attempt to reveal the systematic relationship between *Theodoxus prevostianus* (C. Pfeiffer, 1828) and *Theodoxus danubialis* (C. Pfeiffer, 1828) (Mollusca, Gastropoda, Neritidae)

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## > Abstract

*Theodoxus prevostianus* is a rare and endangered neritid species, occurring in some hypothermal springs in the Pannonian biogeographical region. Recent molecular phylogenetic evidences, based on mitochondrial COI sequences, have questioned its distinct taxon status. Not least because of the species' conservation concern, the aim of this study was to clarify the systematic relationship between *T. prevostianus* and its fluvial sister taxon, *T. danubialis*. Morphological evidences seem to argue for maintaining these two taxa as distinct species whereas available molecular data (mitochondrial COI gene sequences) are largely insufficient to refute this. Assuming heterospecificity, we set up an evolutionary scenario which explains the polyphyletic COI gene tree, and reconcilable with the biogeographical history of the species group. According to this hypothesis, the common ancestor of the two taxa might be the ancient *T. prevostianus*, which lived in the Pleistocene and was quite heterogeneous morphologically as fossil evidences show. Recent *T. prevostianus* populations can be remnant lineages of the stem species, whereas the *T. danubialis* can be one of the lineages that rapidly evolved into a new species. This could be followed by multiple introgressions that confused the original picture, resulting that now, also *T. danubialis* is distributed across more than one lineage.

## > Key words

*Theodoxus*, *danubialis*, *prevostianus*, Pannonian, COI, molecular phylogeny, radula, operculum, morphology.

## Introduction

*Theodoxus*, a freshwater genus of the generally marine family Neritidae, is distributed in the former Thetys-Parathetys area. One of its rarest species is *T. prevostianus* (C. Pfeiffer, 1828), occurring only in a few hypothermal springs within the Pannonian biogeographical region. Due to its special habitat preference and vulnerability, a severe decline was recorded in the past 50 years, thus now, only four remaining populations are known. The species is of high conser-

vation concern therefore; it is listed by the Annex IV of the European Habitat Directive and categorized as endangered, according to IUCN categories (SÓLYMOS & FEHÉR 2007).

BUNJE & LINDBERG (2007) recently investigated the phylogeny and the biogeographical history of European *Theodoxus* species by mitochondrial 16S and COI genes. They have shown that the fluvial *T. danubialis* (C. Pfeiffer, 1828) is the closely related sister species

of *T. prevostianus*. A more detailed study (BUNJE 2007) has found that the COI gene tree of these two species is not reciprocally monophyletic. Though BUNJE (2007) discussed a couple of possible explanations for this phenomenon, and mentioned consistently “lineages” and kept clear of drawing taxonomic conclusions, one might interpret his result as an evidence for the incorrect systematic judgment of the above species, i.e. *T. prevostianus* is either an aggregate of at least two species or *T. prevostianus* and *T. danubialis* are conspecific.

If any of the above interpretations were true – apart from the theoretical importance – that would entail the alteration of the conservation concern of the *T. prevostianus* (e.g. IUCN categories, Annex status, etc.). Therefore, our aim was to reveal the conspecific and/or heterospecific relations within the group. First, we have reviewed the biogeographical past of the species group, based on available fossil data. Second, we have studied those morphological features which are considered meaningful in species distinction among neritids: shell morphology (fossil and recent), as well as radula and operculum morphology (recent). And finally, we tried to reconstruct the species group’s phylogeny using the same gene sequence as BUNJE (2007), but adding samples from a wider geographical range and applying alternative analyzing methods, not least to test the robustness of the phylogenetic signal.

## Material and methods

### Collection and examined material

For shell-, operculum- and radula morphology, *T. danubialis* and *T. prevostianus* material, housed in the Hungarian Natural History Museum Budapest (HNHM), Hungarian Geological Institute Budapest (HGIB), Naturmuseum Senckenberg Frankfurt/Main (NSF), Naturhistorisches Museum Basel (NHB), Museum für Naturkunde Berlin (NMB), Natural History Museum of Zagreb (NHMZ), Phyletisches Museum Jena (PMJ), Museum für Tierkunde Dresden (MTD) and in the private collection of M. L. Zettler (MLZ) were studied by the second author.

For the molecular study, samples were collected from 2005 to 2007. Sampling was extended to each known *T. prevostianus* populations (including that of Răbăgani before its extinction) and to the eastern portion of the geographical range of *T. danubialis*. Sampling localities are listed in Table 1. and shown on the map of Fig. 1. The specimens, fixed and preserved in 96% ethanol, have been deposited in the Mollusca Collection of the HNHM.

### DNA extraction, amplification and sequencing

In total, 30 *Theodoxus danubialis* and 17 *Theodoxus prevostianus* specimens were studied. DNA was extracted according to the modified CTAB protocol, based on DOYLE & DOYLE (1987). A 658 bp fragment of the mitochondrial gene cytochrome oxidase subunit I (COI) was amplified by polymerase chain reaction (PCR) using the primers LCO 1490 and HCO 2198 (FOLMER et al. 1994). Two internal primers, F4d (5'-TAC TTT RTA TAT TAT GTT TGG T-3') and R1d (5'-TGR TAW ARA ATD GGR TCW CCH CCV CC-3') (BUNJE 2005) were also used. PCR was carried out according to BUNJE (2005). Successfully amplified products were purified using Ultrafree-DA PCR Purification columns (Millipore), according to the manufacturer’s protocol. Purified PCR products were then sequenced from both directions with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) using the PCR primers given above.

### Phylogenetic analyses

Identical sequences were collapsed into haplotypes. For reasons of comparability, haplotypes were aligned to the available 600 bp long *Theodoxus danubialis* and *Theodoxus prevostianus* sequences (BUNJE 2007, AY771280–82, AY771293–94, AY771303–19) and cut accordingly. New haplotype sequences were deposited in the GenBank (GQ365716–GQ365728). Haplotype codes and frequencies are listed in Table 1. *Theodoxus fluviatilis* (Linnaeus, 1758) was used as an outgroup (AY765334 and AY765344). For reasons of comparability, haplotypes found only by BUNJE (2007), are indicated the same way as in the original publication (not listed in Table 1). Thus, in total, 34 COI haplotypes (26 *T. danubialis*, seven *T. prevostianus* and two *T. fluviatilis*) were analyzed.

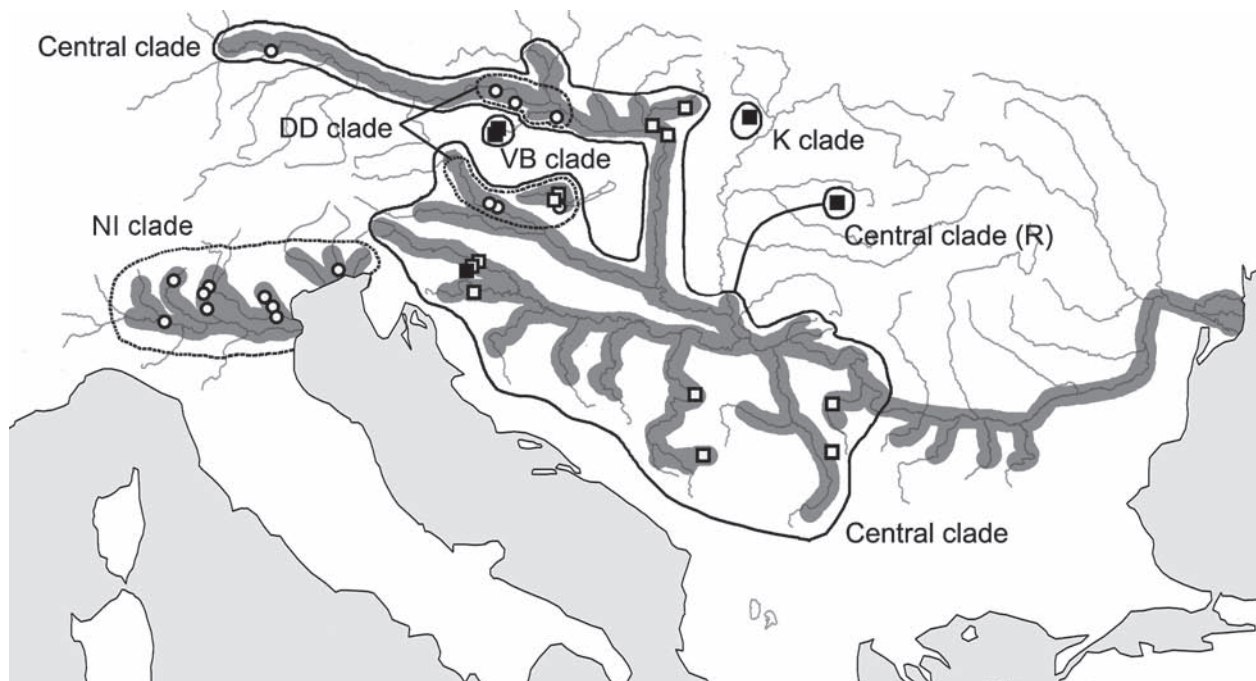
Sequences were aligned by eye, alignment was unambiguous as all the sequences were equal in length and showed an appropriate open reading frame (ORF). The appropriate model for sequence evolution was selected by Modeltest version 3.7 (POSADA & CRANDALL 1998) using Bayesian Information Criterion (BIC). Molecular clock likelihood ratio test (LRT) was performed by Tree-Puzzle version 5.2 (SCHMIDT et al. 2002).

In order to visualize how tree-like is the reconstructed phylogeny, a Neighbor-net network was constructed by SplitsTree4 version 4.10 (HUSON & BRYANT 2006) using an equal angle splits transformation of distances under the HKY model of evolution with 1000 bootstrap replicates.

Phylogenetic trees were constructed by four different methods in order to test the method dependence of

**Tab. 1.** List of sampling localities. Country, region, 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. Note that haplotypes are partly identical with those published by BUNJE (2007).

Country, locality and river	Geographical position	Haplotype	GenBank number	Haplotype frequency
Hungary, Kehidakustány, Zala	46° 50' 11" N 17° 06' 32" E	TDKZ	AY771313	3
Croatia, Karlovac, Kupa	45° 29' 42" N 15° 33' 04" E	TDKZ	AY771313	1
		TDK1	GQ365718	1
Slovenia, Čatež, Sava	45° 53' 34" N 15° 36' 09" E	TDPB	GQ365727	1
		TDC1	GQ365721	1
		TDC3	GQ365722	1
Slovenia, Cerklje, Krka	45° 52' 53" N 15° 31' 15" E	TDPB	GQ365727	2
Austria, Bad Vöslau	47° 58' 03" N 16° 12' 59" E	TPA1	AY771293	1
		TPA2	AY771317	1
Austria, Bad Fischau	47° 49' 54" N 16° 09' 57" E	TPA1	AY771293	2
		TPA2	AY771317	1
Hungary, Kács	47° 57' 49" N 20° 36' 21" E	TPK	AY771294	4
Romania, Răbăgani	46° 45' 04" N 22° 12' 45" E	TPR	GQ365716	5
Slovenia, Bušeča vas	45° 52' 12" N 15° 31' 04" E	TDPB	GQ365728	3
Montenegro, Brodarevo, Lim	43° 12' 47" N 19° 45' 47" E	TDB2	GQ365723	1
		TDD1	GQ365726	1
Serbia, Ovščina, Drina	44° 03' 08" N 19° 36' 50" E	TDD1	GQ365726	4
		TDD2	GQ365724	1
		TDD4	GQ365725	1
Serbia, Gamzigrad, Crni Timok	43° 55' 31" N 22° 07' 46" E	TDT1	GQ365719	1
		TDT3	GQ365720	2
Hungary, Ipolydamásd, Ipoly	47° 50' 30" N 18° 49' 30" E	TDI	AY771281	2
Hungary, Húgyag, Ipoly	48° 05' 38" N 19° 25' 54" E	TDI	AY771281	2
Hungary, Zalacsány, Zala	46° 48' 06" N 17° 06' 54" E	TDKZ	AY771313	2
Hungary, Szentgyörgypuszta, Danube	47° 42' 00" N 19° 06' 00" E	TDI	AY771281	1
Serbia, Jelašnica, Nišava	43° 18' 45" N 22° 03' 12" E	TDJ	GQ365717	2



**Fig. 1.** Location of the study material. Empty symbols: *T. danubialis*, filled symbols: *T. prevostianus*, squares indicate own collectings, circles indicate those of BUNJE (2007). Geographic distribution of the main clades are shown, their names correspond to Fig. 8. Shaded area indicates the recent geographic range of *Theodoxus danubialis*.

the tree topology. (i) An unconstrained Bayesian tree was inferred by MrBayes version 3.1.1 (HUELSENBECK & RONQUIST 2001) using the following parameters: HKY +  $\Gamma$  model of sequence evolution, a four-chain (one cold, three heated;  $T = 0.2$ ) Metropolis-coupled Monte Carlo (MCMC) analysis run for  $10^6$  generations, trees sampled every 100 generations starting after a burn-in of  $10^5$  generations. (ii–iii) Constrained Bayesian trees were constructed using BEAST version 1.4.6 (DRUMMOND & RAMBAUT 2007) with the following settings: HKY +  $\Gamma$  model of sequence evolution with five gamma rate categories, default tree prior and default population size model. Two different relaxed molecular clock assumptions were tested; in which the rate at each branch was drawn from (ii) uncorrelated exponential and (iii) uncorrelated lognormal distributions (DRUMMOND et al. 2006). Following a burn-in of  $10^6$  cycles, every 1000th tree was sampled from  $10^7$  MCMC steps. Convergence of the chains to the stationary distribution was checked by visual inspection of plotted posterior estimates using the program Tracer version 1.3 (RAMBAUT & DRUMMOND 2007). The effective sample size for each parameter sampled from the MCMC analysis was always found to exceed 100. Sampled trees were annotated to a maximum clade credibility tree. (iv) An unconstrained maximum-likelihood (ML) tree was constructed using Tree-Puzzle version 5.2 (SCHMIDT et al. 2002) with the following settings: HKY +  $\Gamma$  model of sequence evolution with eight gamma rate categories and quartet puzzling tree search algorithm with 1000 puzzling steps.

Based on the ML tree topology, we utilized the likelihood mapping method of STRIMMER & VON HAESLER (1997) as implemented in Tree-Puzzle version 5.2 (SCHMIDT et al. 2002) to investigate the support of internal branches between the main clades. As this method can manage maximum four clusters, first the whole dataset and then the “central clade” were divided into four clusters (see Fig. 7.).

## Results

### Distribution

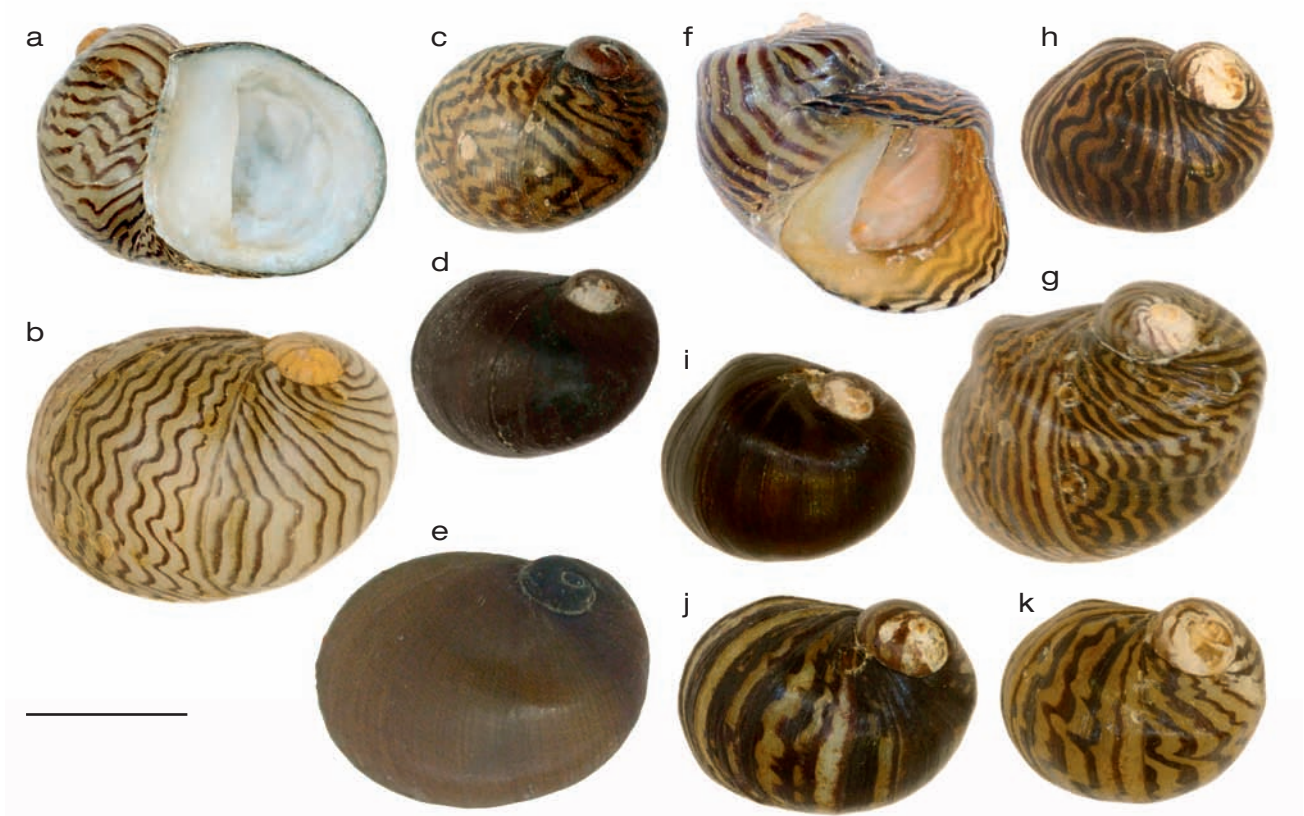
At present, we know about four remaining *T. prevostianus* populations: those of Bad Vöslau and Bad Fischau in Austria, Bušeča vas in Slovenia and Kács in Hungary (Fig. 1.). Several populations went extinct in the recent years, like those of Tata (Fényes-források, Tóváros, Angolkert), Sály (Latori-vízfő), Miskolc-tapolca, Diósgyőr and Budapest (Római-fürdő) in Hungary, Velika, Podsused and Ivanec Bistranski in Croatia and Răbăgani in Romania (FRIVALDSZKY 1865,

GAGIU 2004, JURCSÁK 1969, KORMOS 1905, 1906a, LUKÁCS 1959, PIRINGER 2001, SCHRÉTER 1915, SÎRBU & BENEDEK 2009, SOÓS, 1933, VARGA et al. 2007, VÁSÁRHELYI 1957, WAGNER 1927, 1937). It is a question of debate when did the population of Baile 1 Mai [= Püspökfürdő] in Romania go extinct, either at the end of the 19th century or earlier, but it was certainly a Late Holocene event (BRUSINA 1902, KORMOS 1904, 1905 vs. MOCSÁRY 1872, SOÓS 1906). There are other distribution records in the literature or in the examined collections (Drechselhäuschen in the Bélai Mts.; Secu in Calimani Mts.; Sabljari in Bosnia; Ak-Bunar in Dobruja) but those are either unconfirmed or incorrect (HAZAY 1885, SOÓS, 1943, WAGNER 1942). Each of the confirmed distribution records is located within the Pannonian Basin.

*T. danubialis* is recently distributed within the Danube drainage and in some North Italian lakes and rivers (ANGELOV 2000, BERAN 2002, BODON & GIOVANELLI 1995, FRANK 1982, GLÖER 2002, KARAMAN 2005, LISICKY 1991, NESEMANN et al. 1997, SÎRBU & BENEDEK 2005) but conspicuously absent from the Tisza river [= Theiss] and its tributaries (see Fig. 1.). Some literature records suggest that its recent range might involve other rivers of the Black Sea drainage, such as Dniepr, Dnester and Bug as well as the Caspian Basin (e.g. EHRMANN 1933, ANDREEV & BURCOVSKI 2004, ZHADIN 1931, 1965), but on the basis of material, originated from these locations, we question the correctness of these records (see also ZETTLER 2007).

Apart from one Miocene record (*T. danubialis pannonicus* Lueger, 1979), there are no evidences that the *T. danubialis* – *T. prevostianus* group has occurred in the Pannonian Basin before the Upper Pliocene (BANDEL 2001, MAGYAR et al. 1999). The typical Lower Pleistocene accompanying fauna indicates that LUEGER's (1979) record might have been incorrectly dated (E. KROLOPP personal communication). First reliable records of a *T. prevostianus*-like species are from the Upper Pliocene formations of Újvidék [= Novi Sad] (KORMOS 1910) and Kravarsko (KRSTIĆ 2006) (>1.8–2.4 my). According to KROLOPP (1977), this should be the common ancestor of the recent *T. prevostianus* and *T. danubialis* species. This form – referred also as *Nerita serratilinea* Jan, 1830 or *Nerita fluviatilis parreysi* Villa, 1841 in the literature (e.g. KORMOS 1905, SOÓS 1906) –, was quite frequent in the rivers of the Pannonian Basin in the Lower Pleistocene (KORMOS 1906b, 1910, KROLOPP 1976). Supposed to be identical with *Theodoxus serratiliformis* Geyer, 1914 and *Theodoxus cantianus* Kennard & Woodward, 1924 (see e.g. GLÖER 2002, GITTENBERGER et al. 2004, ZETTLER 2008), in the Pleistocene, this form could be widely distributed from England, Southern Germany and Poland to the Lower-Danube (CHEPALIGA 1967). Ca. 600.000 years before present, this ancient form





**Fig. 2.** *Theodoxus danubialis* (C. Pfeiffer, 1828): **a–b**) Danube river near Budapest, Hungary, leg. Drimmer, 1986 [HNHM]; **c–d**) Crni Timok river near Gamzigrad, Serbia, leg. Dányi, Kotschán & Murányi, 2006 [HNHM]; **e**) Ipoly river near Ipolydamásd, Hungary, leg. Jueg, Fehér & Glöer, 2003 [MLZ]; **f–g**) Kupa river near Karlovac, Croatia, leg. Fehér & Tamás, 2006 [HNHM]; **h–k**) Sava river near Čatež, Slovenia, leg. Fehér & Tamás, 2006 [HNHM]. Scale bar = 5 mm.

has immediately disappeared from the Pannonian rivers, younger Pleistocene records can only be found in the tufa layers of some warm springs (Eger, Tata, Szomód, Vértesszőlős, Rontó, Püspökfürdő, Epöl (SCHRÉTER 1915)). Later, in the Holocene, the Pannonian rivers were recolonized by a larger sized form, the recent *T. danubialis*.

### Shell, radula and operculum morphology

The shell of typical *T. danubialis* is hemispherical or semiovoid with a low spire and a blunt apex, consists of  $2\frac{1}{2}$ – $2\frac{3}{4}$  whorls, its surface is regularly and finely lined, the shell length reaches up to 13.6 mm (n = 408 specimens) (Fig. 2.). In some specimens, on the upper part of the ultimate whorl, a more or less conspicuous ridge can be seen. This ridge is especially characteristic to the populations of the Sava and the Drava drainages (Fig. 2. f–k). It is a question of debate whether or not these forms – *T. danubialis stragulatus* (C. Pfeiffer, 1828) and *T. danubialis carinatus* (Schmidt, 1847) – are separate subspecies (see e.g. GLÖER 2002 and NESEMANN et al. 1997). Predominantly, shells have a brownish or purplish zig-zag shaped pattern on a pale-

yellowish or whitish background, but in exceptional cases, black or brown specimens can also be observed. (Fig. 2. d, i). *T. prevostianus* has a smaller and somewhat more elongated shell (up to 9.2 mm, n = 555 specimens), with somewhat more irregularly and more widely lined surface. Remaining populations consist of uniformly inky-black specimens (Fig. 3. a–g), with a magenta lustre or faded patterns in some exceptional cases (Fig. 3. h, j, o). In contrast, the population of Tata, which went extinct in the 1960s, consisted of purple specimens, some of them were uniformly purple, others had zig-zag shaped or faded white patterns (Fig. 3. p–s). It is notable that some specimens of this population were relocated to Budapest (Római-fürdő) (Fig. 3. i–j), where they turned darker and lost their patterns after a few generations (Soós 1933). In the Pleistocene fluvial records (e.g. of Szabadhídvég and Nagornoje, HGIB Coll.), the form with dark zig-zag shaped pattern on a whitish background prevailed, but ca. 1–2 % of the specimens were uniformly dark (Fig. 3. t–aa) and still less frequently, but larger sized specimens occurred as well (this latter form is referred as *T. danubialis* by KROLOPP (2003)).

The radula in both species is rhipidogloss, where the central tooth is flanked on each side by four lateral and numerous marginal teeth. The central tooth

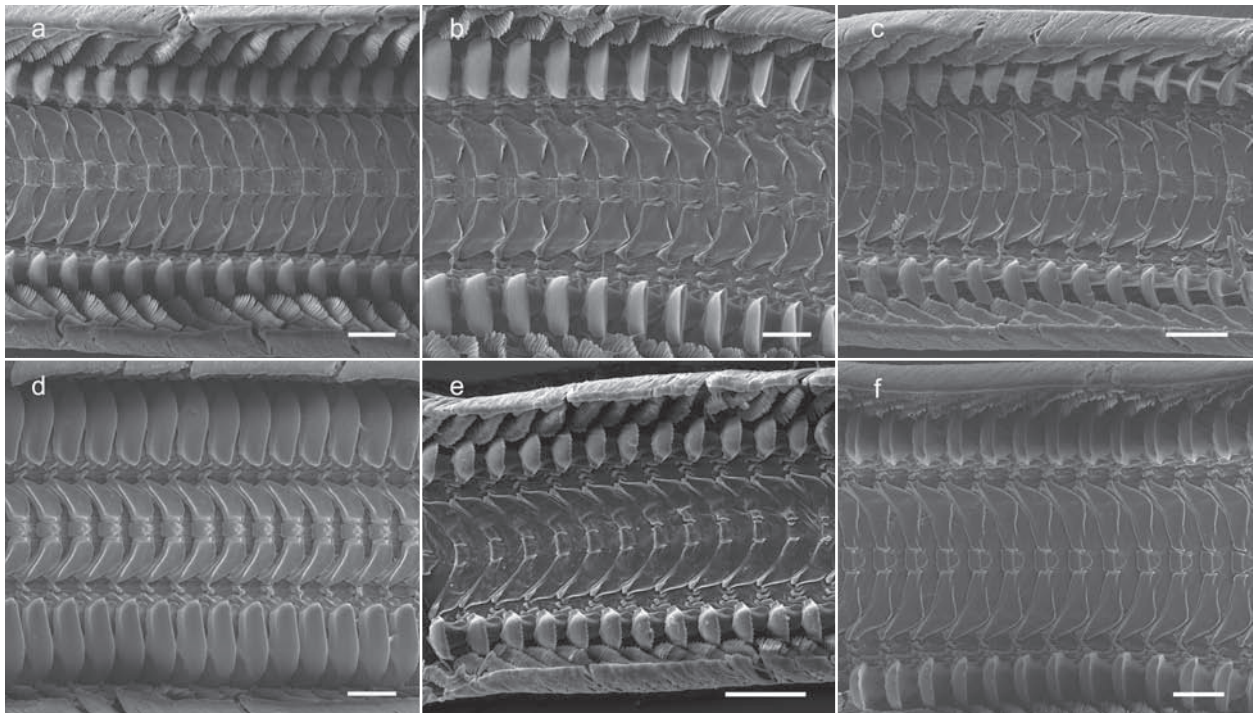


**Fig. 3.** *Theodoxus prevostianus* (C. Pfeiffer, 1828): **a–c**) Kács, Hungary, leg. Fehér & Tamás, 2005 [HNHM]; **d**) Klunove Teplíce spring, Bušeča vas, Slovenia, leg. Fehér & Tamás, 2006 [HNHM]; **e–f**) Hansybach, Bad Vöslau, Austria, leg. Zimmermann [NMB]; **g**) Răbăgani, Romania, leg. Sirbu, 2002 [MLZ]; **h**) Bad Fischau, Austria, leg. Fehér & Tamás, 2006 [HNHM]; **i–j**) Budapest, Római-fürdő, Hungary, leg. Wiesinger [HNHM]; **k–n**) Baile 1 Mai [= Püspökfürdő], Romania (Holocene), leg. Kovács, 1984 [HNHM]; **o**) Kács, Hungary, leg. Drimmer, 1994 [HNHM]; **p–s**) Tata, Angolkert, Hungary [HGIB]; **t–v**) Nagornoje, Danube tributary, Ukraine (Lower Pleistocene) [HGIB]; **w–aa**) Szabadsídvég, Hungary (Lower Pleistocene), leg. Krolopp [HGIB]. Scale bar = 5 mm.

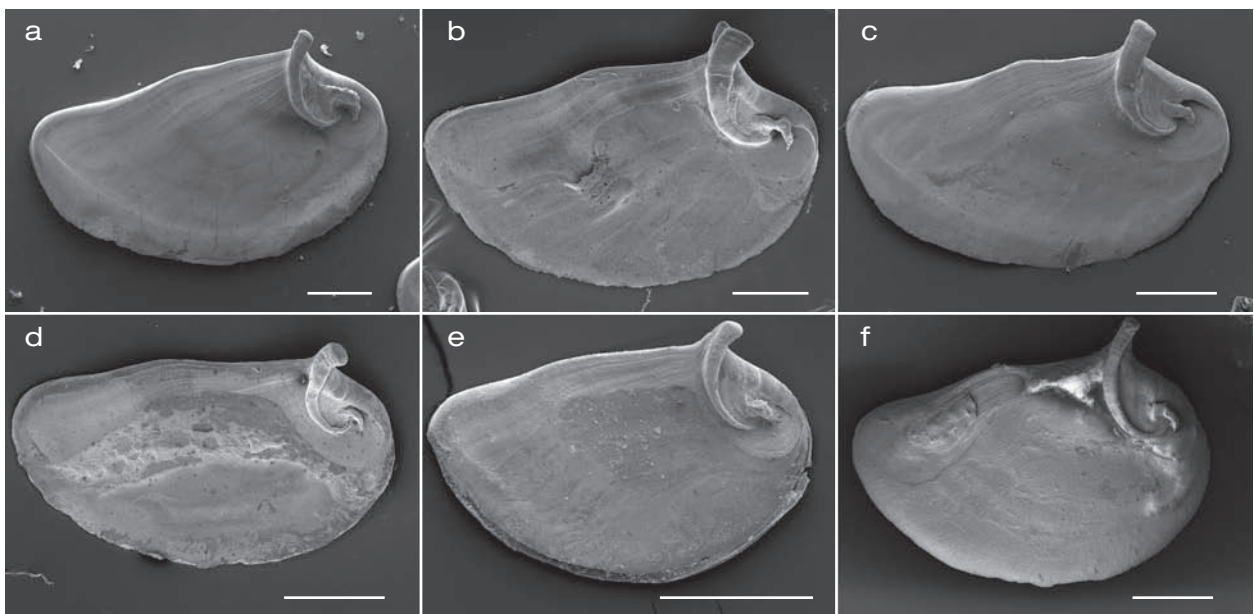
is more or less isodiametric, first and fourth laterals are large, second and third laterals are small, marginal teeth are very minute. The radula of the two taxa are rather similar, they seem to differ only in the shape of the first lateral tooth. In *T. danubialis*, this is more

or less triangular with a pointed angle, reminding to a half harpoon, whereas in *T. prevostianus*, this is more smooth arcuated, reminding to a boomerang (Fig. 4.). As the size and the structure of the radula is rather constant throughout the European *Theodoxus* species





**Fig. 4.** Radula morphology of *T. danubialis* (a–c) and *T. prevostianus* (d–f). **a**) Ipoly river near Ipolydamásd, Hungary, leg. Jueg, Fehér & Glöer, 2003 [MLZ]; **b**) Leitha river near Gattendorf, Austria, leg. Falkner & Neumann, 1991 [MLZ]; **c**) Caraş river near Gradinari, Romania, leg. Sîrbu, 1998 [MLZ]. **d**) Kács, Hungary, leg. Drimmer, 1994 [HNHM]; **e**) Bad Vöslau, Austria, leg. Zimmermann [NMB]; **f**) Klunove Teplice spring, Bušeča vas, Slovenia, leg. Hirschfelder, 2003 [MLZ]. Scale bar = 100  $\mu$ .

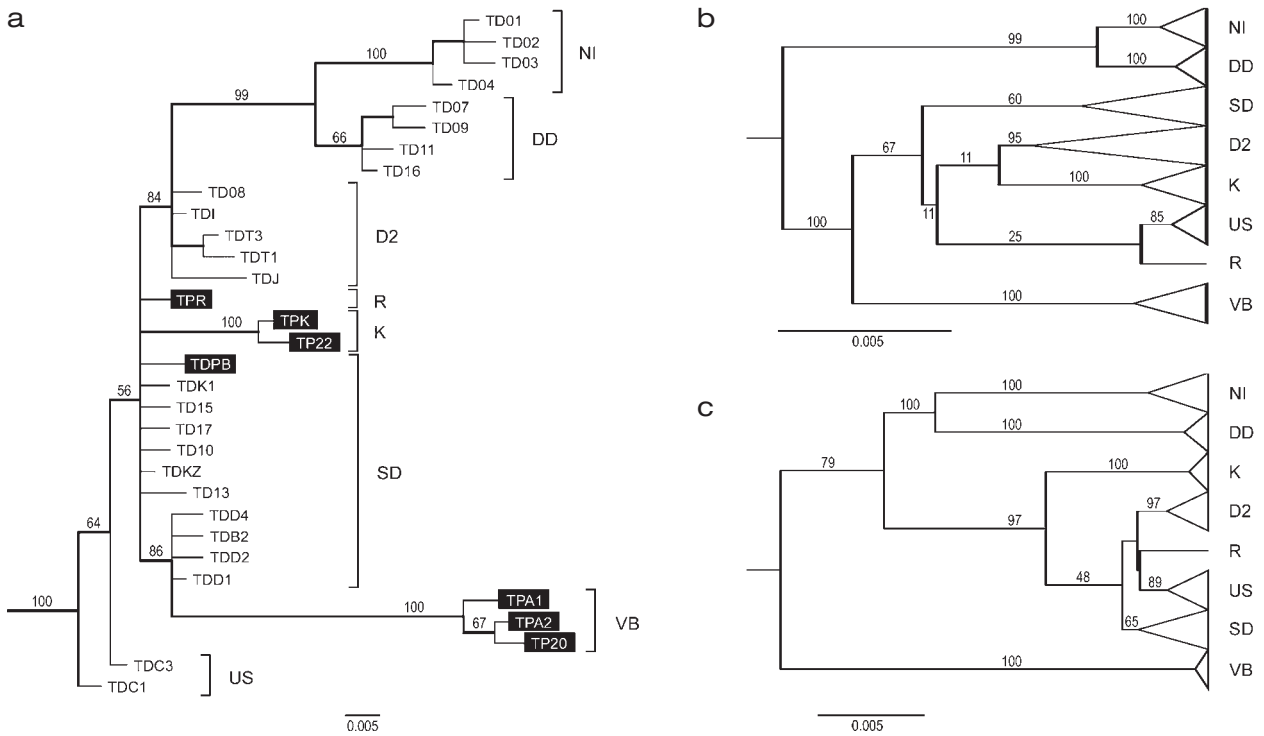


**Fig. 5.** Operculum morphology of *T. danubialis* (a–c) and *T. prevostianus* (d–f). **a**) Ipoly river near Ipolydamásd, Hungary, leg. Jueg, Fehér & Glöer, 2003 [MLZ]; **b**) Leitha river near Gattendorf, Austria, leg. Falkner & Neumann, 1991 [MLZ]; **c**) Caraş river near Gradinari, Romania, leg. Sîrbu, 1998 [MLZ]. **d**) Kács, Hungary, leg. Drimmer, 1994 [HNHM]; **e**) Bad Vöslau, Austria, leg. Zimmermann [NMB]; **f**) Klunove Teplice spring, Bušeča vas, Slovenia, leg. Hirschfelder, 2003 [MLZ]. Scale bar = 1 mm.

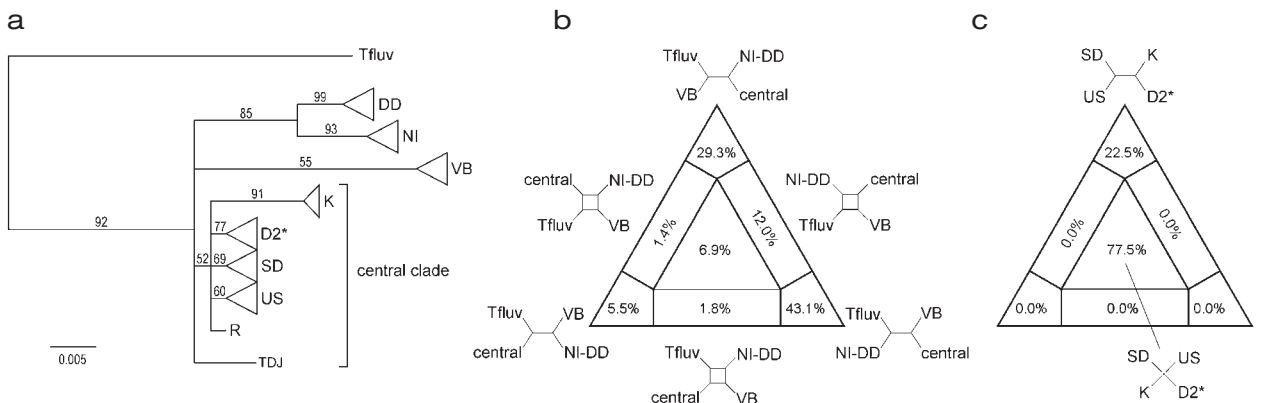
(except for *T. transversalis*) (ZETTLER 2008), even the observed small differences might have significance to distinguish the two species.

The opercula of these two species are also similar. The colour is pale yellow-reddish, sometime more

greyish. One big apophyse (columella) and one more or less slight second apophyse are visible. The only difference is the shape of the second apophyse at the base of the columella; in *T. danubialis* it seems to be more prominent than in *T. prevostianus* (Fig. 5).

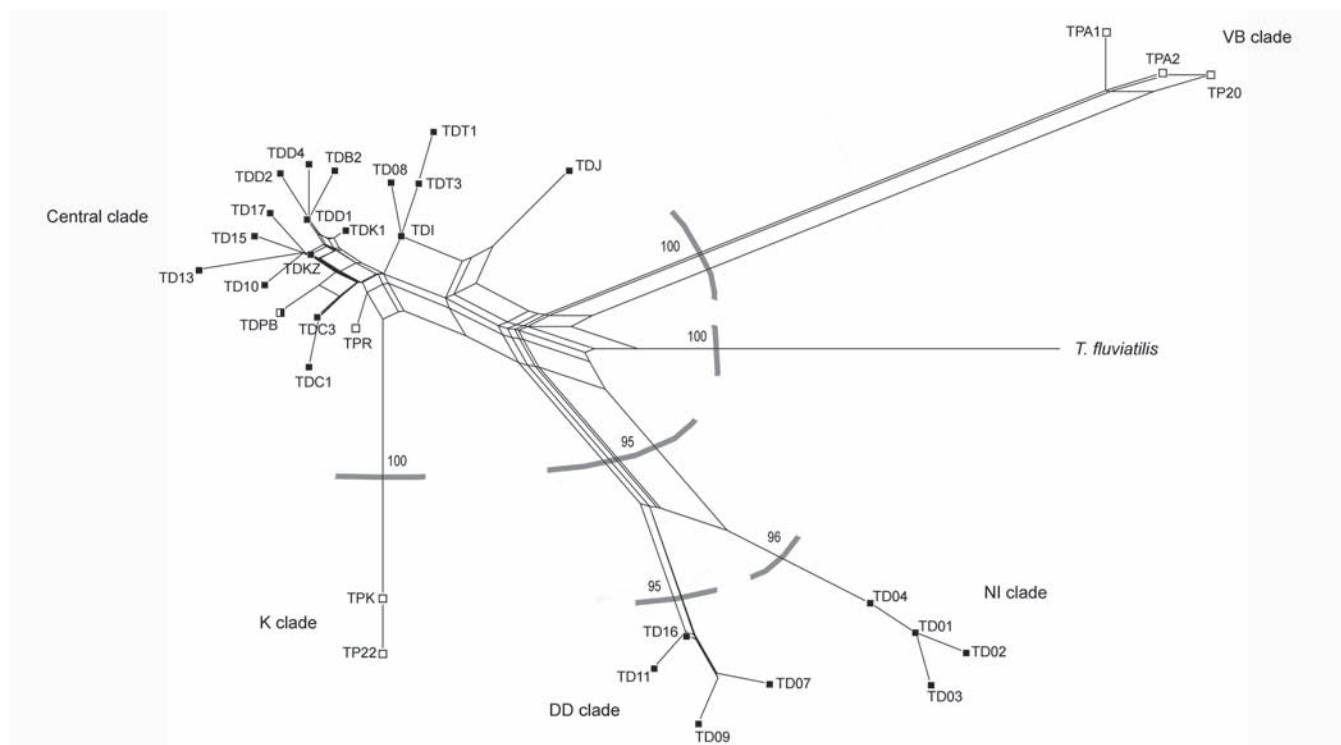


**Fig. 6.** Bayesian COI trees of the *T. prevostianus* – *T. danubialis* group constructed by three different methods. **a)** unconstrained Bayesian tree, **b)** constrained Bayesian tree assuming a relaxed molecular clock model, in which the rate at each branch is drawn from an exponential distribution c.) same as b.) but the rate at each branch is drawn from a lognormal distribution. *T. prevostianus* haplotypes are indicated by white letters on black background, note that TDPB haplotype is shared by both species. For reasons of transparency, main clades of the constrained trees are collapsed. Clade abbreviations are the following: NI, Northern Italy; DD, Danube–Drava drainage; D2, Danube drainage; R, Răbăgani spring; K, Kács spring; SD, Sava–Drava drainage; VB, Vienna Basin; US, Upper Sava drainage. Scale bar indicates the expected number of substitutions per site. Numbers over branches are Bayesian posterior probabilities. Trees are rooted with *T. fluviatilis* (not indicated).



**Fig. 7.** Maximum likelihood (ML) tree and grouped likelihood mapping diagrams of the *T. prevostianus* – *T. danubialis* COI sequences. **a)** ML tree inferred by quartet puzzling algorithm. For reasons of transparency, main clades are collapsed and clade abbreviations correspond to Fig. 6. Note that TDJ haplotype and the rest of the D2 clade of Bayesian trees are not monophyletic here, thus, the latter is indicated as D2\*. Scale bar indicates the expected number of substitutions per site. Numbers over branches are quartet puzzling support values of those branches. **b)** Likelihood mapping diagram of the whole dataset, which is grouped into the following four clusters: *T. fluviatilis* (Tfluv) as outgroup, NI–DD clade, VB clade and the rest of the *T. danubialis* – *T. prevostianus* sequences (= central clade including TDJ haplotype). **c)** Likelihood mapping diagram of the sequences of the central clade (excluding TPR and TDJ haplotypes), grouped into four clusters (SD, US, D2\* and K). Values in the seven areas of the diagram correspond to the percentage of fully resolved (tips) partly resolved (sides) and completely unresolved (middle) quartets.





**Fig. 8.** Neighbor-net network of the COI sequences of the *T. prevostianus* – *T. danubialis* group. Clades supported by more than 50% bootstrap value are indicated, clade names correspond to Fig. 6. Filled symbols: *T. danubialis*, empty symbols: *T. prevostianus*, semifilled symbol indicates the TDPB haplotype which is shared by both species.

### Mitochondrial COI phylogeny

Within the *T. danubialis* – *T. prevostianus* group, 53 of the 600 sites are variable. The average number of pairwise nucleotide differences is 10.34 (= 1.72 %), the largest difference is 24 (= 4 %; TP20–TP22 and TP20–TD03). One of the haplotypes (TDPB) was observed in both species, namely in the *T. danubialis* population of the Krka river and in the *T. prevostianus* population of the Buševca vas spring. The clock hypothesis could not be rejected under the HKY +  $\Gamma$  model of sequence evolution on a significance level of 5 % ( $\log L_0 = 1480.18$ ,  $\log L_1 = 1458.48$ ,  $-2 \log \Lambda = 43.40$ ,  $DF = 32$ ,  $p = 0.0861$ ).

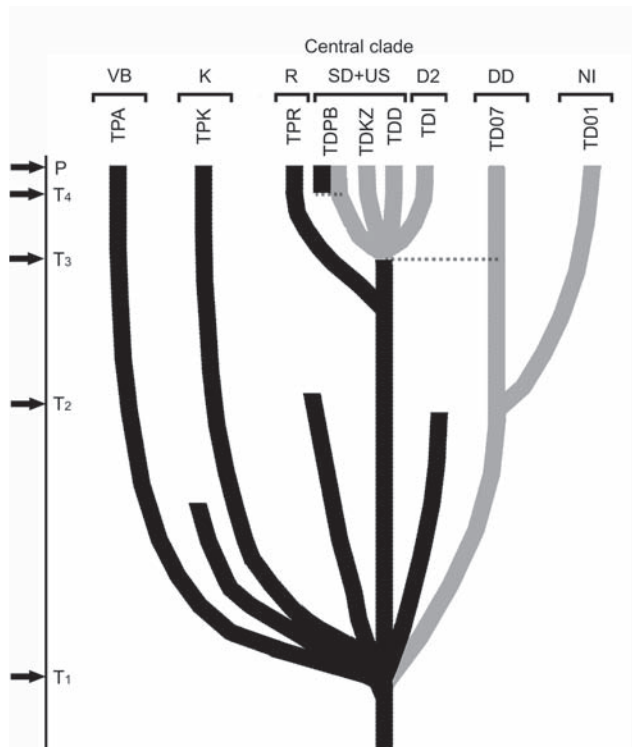
Although the compositions of the terminal clades are more or less the same in the trees (Figs 6–7., see Fig. 6. for their composition and the meaning of their abbreviated names), depending on the settings of the employed tree building methods, overall topologies are different. Apart from the TDPB haplotype, *T. prevostianus* recovered as three distinct lineages. The most notable difference between the four tree topologies is that different lineages are inferred to be the sister group of all others. The NI–DD lineage, the VB lineage and the TDC1 haplotype of the US clade was inferred to be the most basal by the two different constrained and the unconstrained Bayesian methods, respectively (Fig. 6.). ML tree (Fig. 7a.) was unable to resolve the branching order: four lineages are derived

from a basal polytomy and there is a further polytomy at the base of the central clade. As regards overall topology, likelihood mapping analysis inferred the largest support for the topology where NI–DD clade is the most basal within the species group, but not even this value exceeds 50 % (Fig. 7b.). Within the central clade, the completely unresolved tree got far the largest support (77.5 %) (Fig. 7c.).

Neighbor-net network provides a similar topology to that of the ML tree (Fig. 8.), i.e. haplotypes belonging to the so called R, D2, US and SD clades form a “central” group from which VB, K, NI and DD clades stand apart, but their relationships are not clearly resolved.

### Discussion

As the correct taxonomic judgment of *T. prevostianus* has been challenged, we have to check two scenarios, namely that *T. prevostianus* is an aggregate of at least two species and that *T. prevostianus* is conspecific with *T. danubialis*. Considering the morphologically uniform shells, radulae and opercula of different *T. prevostianus* populations as well as the fact that genetic distance between the two most distant *T. prevostianus* COI haplotypes is less than the largest intraspe-



**Fig. 9.** Hypothetic phylogeny of the *T. prevostianus* – *T. danubialis* group. Time is shown on the vertical axis, **T1**: the oldest split between existing lineages, **T2**: split between DD and NI lineages (i.e. the colonization of North Italy), **T3**: when the mitochondrion of the “central clade” introgressed into *T. danubialis*, **T4**: when the mitochondrion of the Upper Sava *T. danubialis* clade introgressed into the *T. prevostianus* population of Bušeča vas, P: present. Grey branches indicate *T. danubialis*, black branches indicate *T. prevostianus* lineages. Dotted vertical lines symbolize proposed introgressions, branches that does not reach up to P symbolize extinct lineages. Only some representative haplotypes are indicated, clade and haplotype names correspond to Figs 6–8. and Table 1.

cific distances within the related *T. fluviatilis* (BUNJE 2005), the former scenario seems to be highly unrealistic. Therefore, if incorrect taxonomy is supposed, we should investigate whether or not *T. prevostianus* and *T. danubialis* are conspecific.

One might believe that TDPB haplotype, shared between *T. danubialis* and *T. prevostianus*, is an evidence for conspecificity. But this phenomenon, i.e. the sympatric sharing of a geographically localized mtDNA haplotype between morphologically divergent species, can also be interpreted by ongoing (or very recent) and geographically localized interspecific gene flow (FUNK & OMLAND 2003). As the spring of Bušeča vas is only a few hundred meters far from the Krka river, it is reasonable that the two populations can contact, and being closely related taxa, they are able to hybridize too. In the Upper Sava region, we have found three different but closely related COI haplotypes in *T. danubialis*, namely TDC1, TDC3 and TDPB. As only the latter one was present in *T. prevostianus*, it suggests that mitochondrial introgression was directed from the *T. danubialis* into the *T. prevostianus*.

The inferred COI trees suggest that *T. prevostianus* and *T. danubialis* are not reciprocally monophyletic. By and large, this finding is in agreement with that of BUNJE (2007), however, due to the widened sampling and different analyzing methods, our results are somewhat different. Polytoamy, inferred by Neighbor-net and ML analyses, might be interpreted in two ways. If this is supposed to be a hard polytoamy, than the present clades evolved from the same ancient form by a star-like radiation event, with largely different mutation rates. There is another explanation, which is just as, if not more probable, namely that the phylogenetic information in the COI gene is insufficient (soft polytoamy). This latter might be one of the possible explanations for the striking difference between Bayesian tree topologies. Though molecular clock hypothesis was not rejected by the LRT, dissimilarities between constrained and unconstrained trees might be due to that faster evolved lineages were incorrectly forced to the basal position in constrained trees. Method dependency of the tree topologies indicates that results are not robust enough. Moreover, a growing body of evidence suggests that individual gene trees are often in conflict with the species tree, therefore, inferences based on any individual gene, a mitochondrial gene in particular, should be interpreted cautiously (FUNK & OMLAND 2003, SPINKS & SCHAFFER 2009). As long as no further gene sequences will be available, we have to rely primarily on fossil records and traditional morphology.

The shell morphology – especially the shell size –, on which the distinction of the two species was based on so far (e.g. MARTENS 1879, GLÖER 2002), seems clearly different. But this is the character, which was questioned to bear any taxonomical relevance (BUNJE & LINDBERG 2007). Assuming conspecificity, the smaller shell size and the uniformly black shell colour of the thermal spring populations might be explained that they are ecological morphs. The most conceivable way to verify or refute this hypothesis would be the relocation of some *T. danubialis* specimens from a fluvial biotope to a hypothermal spring in order to make sure whether their size and colour changed after some generations. Being a protected red-list species, such an experiment on the *T. danubialis* has not only ethical but legal obstacles. Nevertheless, recent and fossil records provide some information about the relationship between shell morphology and the environment and some facts seem to contradict the ecological morph scenario: (i) In the Pleistocene, the small sized „*prevostianus*“-form prevailed in fluvial biotopes whereas the „*danubialis*“-form occurred only rarely and did not became widespread. (ii) Though rarely, but there are uniformly dark specimens also in fluvial biotopes. (iii) Not only black but also zig-zag patterned populations lived in warm springs. (iv) When

zig-zag patterned specimens were relocated from Tata to Budapest (almost the same was the water temperature in both localities), the new colony changed after a few generations. These facts suggest that even if the shell morphology is influenced by the environment, *T. prevostianus* can not be considered simply as the thermal ecotype of *T. danubialis*. Operculum and radula morphology are known to be usable features of the species distinction within the genus (BANDEL 2001, ZETTLER et al. 2004, ZETTLER 2008). It is not easy to assess the significance of the observed slight differences, but they might support the heterospecificity of *T. prevostianus* and *T. danubialis*.

Assuming heterospecificity, we need to set up an evolutionary scenario which explains the polyphyletic COI gene tree, and which is reconcilable with the biogeographical history of the species group. According to our hypothesis, it is conceivable that ca. 600.000 years before present, when the once widely distributed ancient *T. prevostianus* disappeared from the Pannonian rivers, some lineages survived in some refugia (in Pannonian warm springs and in some rivers in the Balkans). Due to genetic drift, the certain lineages of this morphologically heterogeneous species retained different morphological features: small and black shells in some warm spring populations, small and zig-zag patterned shells in some others and large and zig-zag patterned shells in the fluvial refugium. This latter one evolved rapidly into a new species, *T. danubialis*, which then successfully recolonized the Pannonian rivers in the Holocene. Among molluscs, there are several examples for similar cases, when a lineage of a genetically divergent but morphologically uniform stem species evolved to a morphologically well distinct new species, resulting a paraphyletic pattern (e.g. *Bythinella robiciana* from *B. opaca* (HAASE et al. 2007) or *Corbicula anomioides* from *C. moltkiana* (RINTELEN & GLAUBRECHT 2005)). The presumed very recent introgression, detected in the Upper Sava region suggests that such events could happen also in the past. Thus, we hypothesize that additionally, multiple introgressions confused the original picture, resulting that now, also *T. danubialis* is distributed across more than one lineage (Fig. 9.). Of course, it could only be confirmed by a simultaneous analysis of mitochondrial and multiple nuclear markers. It must be admitted that the above scenario is largely speculative, but explains some points that the „imperfect taxonomy“ scenario doesn't.

In summary, morphological evidences argue for maintaining these two taxa as distinct species whereas available molecular data are largely insufficient to refute this. Thus, the synonymization of the two species would be premature and unfounded for now. The regrettable extinction of the Răbăgani population indicates (SÎRBU & BENEDEK 2009) that each *T. prevos-*

*tianus* population is very vulnerable and an imprudent downgrade of the species' protection status might have irreversible consequences. The surviving populations seem to be relicts of at least two, or maybe three divergent lineages of the ancient *T. prevostianus* species. This even increases their conservation concern and therefore, they might be treated as distinct conservation units.

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