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 conservation concern therefore; it is listed by the Annex IV of the European Habitat Directive and categorized as endangered, according to IUCN categories (SOLYMOS & 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 Ribhagani 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).

<table>
<thead>
<tr>
<th>Country, locality and river</th>
<th>Geographical position</th>
<th>Haplotype</th>
<th>GenBank number</th>
<th>Haplotype frequency</th>
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<tr>
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<tr>
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<tr>
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Fig. 1. Location of the study material. Empty symbols: *T. danubialis*, filled symbols: *T. prevostianus*, squares indicate own collections, 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 (HUELENEBECK & ROUQUEST 2001) using the following parameters: HKY + Γ model of sequence evolution, a four-chain (one cold, three heated; T = 0.2) Metropolis-coupled Monte Carlo (MCMC) analysis run for 10^8 generations, trees sampled every 100 generations starting after a burn-in of 10^6 generations. (ii–iii) Constrained Bayesian trees were constructed using BEAST version 1.4.6 (DRUMMOND & RAMBOUT 2007) with the following settings: HKY + Γ 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 + Γ 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 HAASEL (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, Buceca vas in Slovenia and Kács in Hungary (Fig. 1.). Several populations went extinct in the recent years, like those of Tata (Fényes-főrások, Tóváros, Angolkert), Sály (Latori-vízfő), Miskolctapolca, Diósgyőr and Budapest (Római-fürdő) in Hungary, Velika, Podsused and Ivanec Bistranski in Croatia and Răhăgani in Romania (PRVÁLSZKY 1865, GAGIU 2004, JURCSÁK 1969, KORMOS 1905, 1906a, LUKÁCS 1959, PRINGR 2001, SCHÖTER 1915, SIRBU & BENEDEK 2009, SÓOS, 1933, VARGA et al. 2007, VÁSÁRHELYI 1957, WAGNER 1927, 1937). It is a question of debate when did the population of Baile 1 Mai [= Püspöfkúrtő] 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. MOCZÁRY 1872, SÓOS 1906). There are other distribution records in the literature or in the examined collections (Drechselhülsen in the Bélaí Mts.; Secu in Calimani Mts.; Sabljari in Bosnia; Ak-Bunar in Dobruja) but those are either unconfirmed or incorrect (HAZAY 1885, SÓOS, 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, GLOER 2002, KARAMAN 2005, LI-SICKY 1991, NEISEMANN et al. 1997, SIRBU & 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, Donetser and Bug as well as the Caspian Basin (e.g. EHRMANN 1933, ANDREEV & BURCOVSCHI 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 pantonicus LIEUER, 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 LIEUER’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 Újjvidék [= Novi Sad] (KORMOS 1910) and Kravarsko (KRSITIĆ 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 parreysii Villa, 1841 in the literature (e.g. KORMOS 1905, SÓOS 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 serratifoliformis Geyer, 1914 and Theodoxus cantianus Kennard & Woodward, 1924 (see e.g. GLOER 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 (CHEPAŁOJA 1967), Ca. 600.000 years before present, this ancient form
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, Rónút, Püspökfürdő, Épöl (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 2½–2¾ 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

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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

(except for T. transversalis) (ZEITLER 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 or a lognormal distribution. *T. prevostianus* haplotypes are indicated by white letters on a 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, Ráhúgári 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 TDI 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* (TFuv) as outgroup, NI–DD clade, VB clade and the rest of the *T. danubialis* – *T. prevostianus* sequences (= central clade including TDI haplotype). **c**) Likelihood mapping diagram of the sequences of the central clade (excluding TPR and TDI 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.
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 Bušeča vas spring. The clock hypothesis could not be rejected under the HKY + Γ 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-
from the suggests that mitochondrial introgression was directed
only the latter one was present in
T. danubialis
three different but closely related COI haplotypes in
tact, and being closely related taxa, they are able to hy-
river, it is reasonable that the two populations can con-
vas is only a few hundred meters far from the Krka

One might believe that TDPB haplotype, shared
between T. danubialis and T. prevostianus, is an evi-
dence 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 re-
cent) 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 con-
tact, and being closely related taxa, they are able to hy-
bридize 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
BUNIE (2007), however, due to the widened sampling
and different analyzing methods, our results are some-
what different. Polytomy, inferred by Neighbor-net
and ML analyses, might be interpreted in two ways.
If this is supposed to be a hard polytomy, than the
present clades evolved from the same ancient form by
a star-like radiation event, with largely different muta-
tion 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 poly-
tomy). This latter might be one of the possible expla-
nations 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 mor-
phology.

The shell morphology – especially the shell size –,
on which the distinction of the two species was based
on so far (e.g. MARTENS 1879, GLOER 2002), seems
clearly different. But this is the character, which was
questioned to bear any taxonomical relevance (BUN-
IE & 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 conceiv-
able 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 re-

Fig. 9. Hypothetic phylogeny of the T. prevostianus – T. danu-
bialis group. Time is shown on the vertical axis, T1: the old-
est split between existing lineages, T2: split between DD and
NI lineages (i.e. the colonization of North Italy), T3: when the
mitochondrium of the “central clade” introgressed into T. danu-
bialis, T4: when the mitochondrium of the Upper Sava T. danu-
bialis clade introgressed into the T. prevostianus population of
Bušeča vas, P: present. Grey branches indicate T. danubialis,
black branches indicate T. prevostianus lineages. Dotted verti-
cal lines symbolize proposed introgressions, branches that does
not reach up to P symbolize extinct lineages.Only some repre-
sentative haplotypes are indicated, clade and haplotype names
 correspond to Figs 6–8 and Table 1.
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* cannot 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 from 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 anomioïdes* 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 hypothesise 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 Râhâgani population indicates (Sirbu & Benedek 2009) that each *T. prevosti-
Tethys marine basin development. – Molecular Phylogenetics and Evolution 42: 373–387.


