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Fluvial range expansion, allopatry, and parallel evolution in a Danubian snail lineage (Neritidae: Theodoxus)

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The snails *Theodoxus danubialis* and *Theodoxus prevostianus* form a single clade native to freshwaters of south-eastern Europe whose inter- and intraspecific relationships remain unresolved. The present study utilized a phylogeographical approach to clarify the relationship of these species as well as to reconstruct the evolutionary and demographic history of populations in the western portion of their range. Phylogenetic, population genetic, and nested clade analyses reveal a clade that has distributed itself upriver from a lower Danube River source population and become genetically distinct primarily through range expansion and localized allopatric divergence. Notably, this geographical pattern is replicated phylogenetically in the form of two cytochrome *c* oxidase subunit I (CO I) lineages that are present simultaneously in individual snails. Haplotypes from polymorphic individuals form two distinct clades, both of which show phylogenetic and nucleotide substitution patterns consistent with a mitochondrial origin, and whose common ancestor must have occurred in a lower Danube source population. Separated allopatrically from their Danubian relatives, populations of *T. danubialis* in northern Italy have also undergone substantial range expansion, much more recently than Danube watershed lineages. In addition to repeated patterns of range expansion, parallelism is found in *T. prevostianus*, which is shown to be a nonmonophyletic taxon of remarkable morphological and ecological similarity. *Biological Journal of the Linnean Society*, 2007, **90**, 603–617.

ADDITIONAL KEYWORDS: heteroplasmy – mitochondrial DNA – nested clade analysis – Pannonian basin – parallelism – phylogeography – range expansion – speciation.

INTRODUCTION

A common feature of phylogeography is that specific habitat types result in different intraspecific patterns of lineage divergence, extinction, and geographical distribution (Avise, 2000; Criscione, Poulin & Blouin, 2005; Emerson & Hewitt, 2005; Lourie, Green & Vincent, 2005). Freshwater habitats provide a unique geographical context for intraspecific lineage evolution because, for primarily riverine species, they are one-dimensional. The linear environment of rivers, streams, and small connected lakes thus provides an environment in which species are expected to migrate along restricted fluvial paths, thereby reducing the types of haplotype distributions that are found (Avise, 2000). Unidirectional range expansion, lineage branching associated with river branching, and long-distance colonization or allopatric divergence associated with distinct drainages are expected to be among the dominant patterns present in the genealogical history of such fluvial species (Bernatchez & Wilson, 1998; Durand, Persat & Bouvet, 1999a; Bernatchez, 2001; Salzburger *et al*., 2003; Johnson, 2005). To better understand how these various patterns are manifested in invertebrate freshwater organisms and how this unique geographical context affects species formation, phenotypic evolution, and genotypic evolution, I have performed a phylogeographical analysis of the related European freshwater snail taxa *Theodoxus danubialis* (Pfeiffer) and *Theodoxus prevostianus* (Pfeiffer).

Theodoxus danubialis, as its name implies, is native to the Danubian river system of eastern Europe. Its primary range includes the Danube River and major

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tributaries in the upper portion of the Danube drainage in Germany, Austria, the Czech Republic, Slovakia, Hungary, Slovenia, Croatia, and Romania. In addition, it has been described from other Balkan states, including northern Greece, and Italy. All members of this genus are sexually reproducing, dioecious snails, which lay benthic egg capsules that hatch a single juvenile egg (Fretter & Graham, 1962; Orton & Sibly, 1990). Individuals survive on hard benthic substrates, typically rocks, in calcium-rich waters (Fretter & Graham, 1962). In the present study, I investigate its distribution in the related drainages of the western Danube watershed. This area includes three major river networks, all of which articulate in the lower Danube of south-eastern Hungary. The intraspecific patterns of haplotype relationships are analysed to clarify the relationships between these drainages and to investigate the possibility of upstream range expansion in a branching fluvial system. To complement these data, I also obtained samples from lakes and rivers in northern Italy where populations of *T. danubialis* may also result from range expansion.

The distribution of *T. danubialis* in northern Italy is not known historically and may represent a recent introduction. Bodon & Giovannelli (1995) hypothesized that Italian populations represent a recent (historical) invasion of Italy from populations in eastern tributaries of the Adriatic Sea. However, it is also possible that much older samples of *T. danubialis* in Italy had simply been overlooked or misidentified as *T. fluviatilis* (L.). These two species are often difficult to distinguish in northern Italy and the systematics of Italian populations has seen little investigation. The present study evaluates the hypothesis of a recent invasion.

Theodoxus prevostianus is a restricted endemic found in only a few thermal springs surrounding the Pannonian basin (Lueger, 1979; Pintér, Richnovszky & Szigethy, 1979). It is an easily distinguishable snail characterized by a globular shape, inky black shell, small size (< 10 mm), and restriction to thermally stable (~24 C) calcareous springs surrounding the Pannonian basin (Pintér *et al*., 1979; Piringer, 2002). These habitats also possess other endemic gastropods, making it likely that these springs have persisted for a significant length of time. Their distinctive biology and recognizable morphology have made *T. prevostianus* a commonly identified member of these habitats in fossil assemblages dating back to the Miocene (Lueger, 1979), thus representing some of the oldest identified members of the *T. danubialis* group. A phylogenetic analysis of *Theodoxus* has found that *T. prevostianus* is most likely a close relative or member of the *T. danubialis* lineage (Bunje, 2004). Phylogeographical methods do not require that only intraspecific data be analysed, but simply that a phylogenetic hypothesis exists for a related group of organisms (Avise, 2000). Therefore, I analyse the entire *T. danubialis*/*T. prevostianus* clade as one unit. Using detailed geographical sampling from populations of both *T. danubialis* and *T. prevostianus*, I describe the role of restricted range expansion and localized allopatry in producing delineate patterns of intraspecific population divergence and morphological evolution.

MATERIAL AND METHODS

Specimen collection and sampling design

To assess the relationships of populations in close proximity, samples were collected from many lakes and rivers within a small geographical area in the glacial lakes and lowland rivers of northern Italy. Samples were also collected from throughout the upper Danube river system. Figure 1 shows the locations from which samples were collected. The geocoded localities are described in Table 1. The sampling design covers much of the range of *T. danubialis*, with dense sampling in certain areas to assess the geographical limits of haplotype diversity.

Theodoxus prevostianus samples were collected from three known populations: two in Austria, and one in Hungary. All other historic populations in Hungary (Pintér *et al*., 1979) were also checked for samples but were found to be so highly modified that they did not generally contain any macrofauna. Various reports that *T. prevostianus* has been found in Romania and Greece could not be verified. Because of their unique biology and restricted distribution, more samples ($N = 15$) were sequenced for each population than for *T. danubialis* (average 5.6 samples per population) to capture more haplotype diversity.

DNA extraction, amplification, and sequencing

Some 15–35 mg of foot and/or head tissue was used to extract genomic DNA using the EasyDNA Tissue Kit (Qiagen). Partial sequences of the mitochondrial gene cytochrome *c* oxidase subunit I (CO I) were amplified by polymerase chain reaction (PCR); 600 bp of CO I were amplified using the primers F4d, 5′-TACTTTR TATATTATGTTTGGT-3′, and R1d, 5′-TGRTAW ARAATDGGRTCWCCHCCVCC-3′. Each 50 L PCR reaction included 1 L genomic DNA, 10 pmol of each primer, 3 nmol dNTPs, 5 L of 10 × PCR buffer (Applied Biosystems), 125 nmol $MgCl_2$, and 1 unit AmpliTaq Gold DNA polymerase (Applied Biosystems). The cycling parameters were an initial cycle of 95 for 10 min followed by 36 cycles of denaturation at 95 for 50 s, annealing at 54 for 1 min, and extension at 72 for 1 min. PCR was completed with a 7 minute final extension at 72 . PCR products were purified

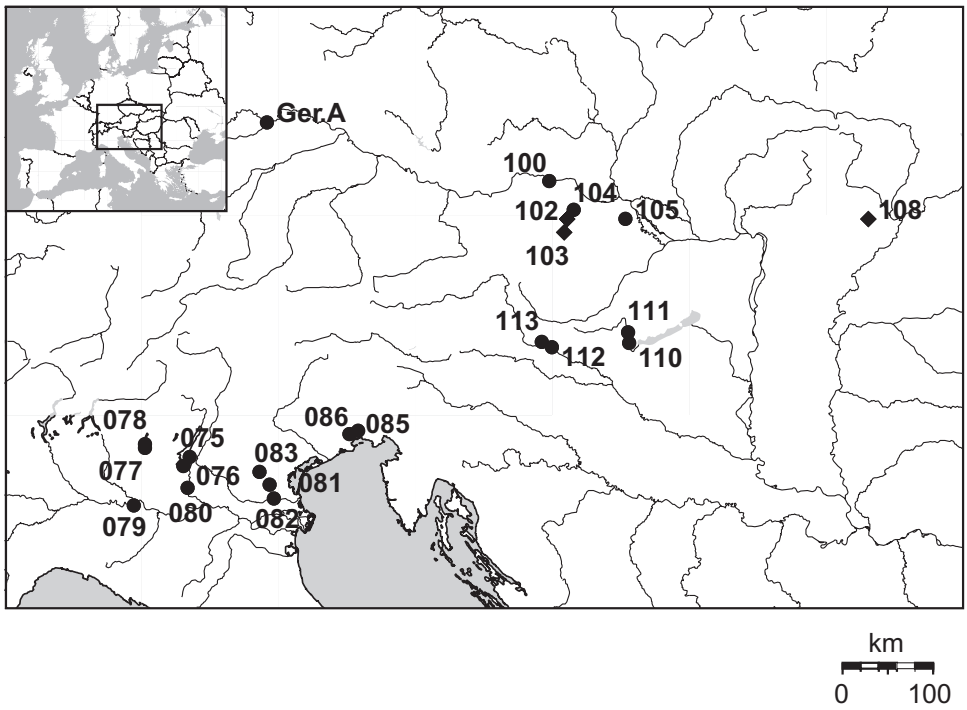

Figure 1. Collection sites for *Theodoxus danubialis* (and *Theodoxus prevostianus*) in central and southern Europe. ◆, locations of *T. prevostianus*; ●, populations of *T. danubialis*. See Table 1 for exact localities and Table 2 for haplotype frequencies.

Table 1. Collection sites of specimens used for the analysis of *Theodoxus danubialis* and *Theodoxus prevostianus*

Site	Water body	Country	Latitude	Longitude
075	Lago di Garda	Italy	45 34.345′	10 42.546′
076	Lago di Garda	Italy	45 28.670′	10 36.640′
077	Lago d'Iseo	Italy	45 39.818′	10 03.011′
078	Lago d'Iseo	Italy	45 42.090′	10 03.109′
079	Po River	Italy	45 04.575′	9 53.543′
080	Mincio River	Italy	45 15.236′	10 40.502′
081	Canale di Cagnola	Italy	45 17.140′	11 52.218′
082	Adige River	Italy	45 08.608′	11 56.089′
083	Tesano River	Italy	45 25.109′	11 43.486′
085	Small stream in Muzanne	Italy	45 49.125′	13 07.768′
086	Canale Cragno	Italy	45 48.382′	13 04.288′
100	Perschling River (Danube)	Austria	48 19.767′	15 57.175′
102	Hansybach	Austria	47 57.986′	16 12.858′
103	Bach Warme Fischau	Austria	47 49.920′	16 10.300′
104	Wiener Neustadter Kanal	Austria	48 03.111′	16 18.681′
105	Leitha River	Austria	47 57.556′	17 04.330′
108	Stream through Kàcs	Hungary	47 57.521′	20 36.778′
110	Zala River	Hungary	46 43.645′	17 07.300′
111	Zala River	Hungary	46 50.177′	17 06.540′
112	Drauchen Bach	Austria	46 41.333′	15 59.704′
113	Gnasbach	Austria	46 44.448′	15 50.715′
Ger.A	Danube River	Germany	48 54.000′	11 50.000′

using QiaQuick PCR Purification columns (Qiagen) or ExoSAP-IT enzyme buffer (USB). Purified PCR products were then sequenced with BigDye, version 2.0, from Applied Biosystems using the PCR primers and visualized on an ABI 377. Both strands were sequenced to ensure the accuracy of the final sequence.

Several specimens from six different localities in Austria and Hungary (although from distinctly different drainages: sites 100, 105, 110, 111, 112, and 113) possessed multiple CO I haplotypes. Haplotypes were determined for polymorphic individuals by subcloning with a Zero Blunt TOPO PCR subcloning kit (Invitrogen) and sequenced using the provided T3 and T7 primers.

Phylogeographical analyses

Gene trees were estimated using phylogenetic analyses implemented in PAUP*, version 4.0b10 (Swofford, 2000). A maximum parsimony (MP) analysis was performed using default settings and an heuristic search strategy with tree bisection reconnection (TBR) branch swapping and ten random addition-sequence replicates. Additionally, maximum likelihood (ML) was employed using unique haplotypes only. After all duplicate haplotypes were culled, ModelTest, version 3.06 (Posada & Crandall, 1998) was used to estimate the best model of nucleotide substitution. Using the chosen model for DNA sequence evolution (TrN + Γ), a likelihood analysis was performed in PAUP* with an heuristic search strategy employing TBR branch swapping and ten random addition-sequence replicates.

Support for reconstructed branches was estimated by ML bootstrapping with 1000 bootstrap replicates and the 'fast' heuristic search algorithm in PAUP*. Bayesian posterior probabilities were also estimated for reconstructed nodes. Bayesian analyses were performed in MrBayes, version 3.0 (Huelsenbeck & Ronquist, 2001) with the following parameters: the model of evolution chosen by ModelTest, a four-chain (one cold, three heated; $T = 0.2$) metropolis-coupled Monte Carlo analysis run for 10^6 generations, trees sampled every 100 generations starting after a burn-in of 50 000 generations. The tree was rooted using an outgroup from *T. fluviatilis*.

A nested clade phylogeographical analysis (NCPA; Templeton, Routman & Phillips, 1995) was performed for the entire *T. danubialis*/*T. prevostianus* group. Construction of a haplotype network employed a statistical parsimony procedure (Templeton, Crandall & Sing, 1992) implemented in the program TCS, version 1.13 (Clement, Posada & Crandall, 2000). The networked haplotypes were then nested into hierarchical clades following the nesting rules outlined in Templeton, Boerwinkle & Sing (1987) and extended in Templeton & Sing (1993). The statistical test of geographical association among and between haplotypes and nested clades was performed using GeoDis, version 2.0 (Posada, Crandall & Templeton, 2000) run with 10 000 permutations. Finally, the updated inference key of Templeton (2004) was used to infer particular historical processes for clades where significant geographical association or dispersion was found.

Haplotypes from polymorphic individuals showed no signs of a nuclear origin and the phylogenetic topology is inconsistent with the presence of a haplotypic lineage that has been captured by the nucleus (see Results), leading to the conclusion that all haplotypes from polymorphic individuals are mitochondrial. Therefore, each time a particular haplotype was sampled, it was considered to be present in that population equal to the number of times it was directly observed. This treatment serves to accurately represent the number of times a particular haplotype is present in a given population.

Standard nucleotide (π) and haplotype (H) diversities (Nei, 1987) were computed using the program Arlequin, version 2.001 (Schneider, Roessli & Excoffier, 2000). To test the hypothesis of Bodon & Giovanelli (1995) that *T. danubialis* in Italy represents a recent colonization, the pairwise distribution test of Rogers & Harpending (1992) was performed on the entire clade and on just the subset that included the Italian samples. This test determines the probability that the observed mismatch distribution comes from a population having undergone recent population growth (i.e. is unimodal) by comparison with a randomized distribution of the observed data using a parametric bootstrap under a model of sudden demographic expansion (Slatkin & Hudson, 1991). A mismatch distribution was also calculated for all other monophyletic groups found by the phylogenetic analysis to determine what other areas of the range, if any, have also experienced recent population expansion.

RESULTS

Phylogenetic analysis

CO I sequences were obtained for 152 individuals: 45 *T. prevostianus* and 107 *T. danubialis*. This resulted in 21 unique haplotypes, including 11 singletons; GenBank accession numbers are shown in Table 2. Twelve different haplotypes were found from 18 subcloned individuals. One population displayed six unique haplotypes and one individual had five different haplotypes in its genome (out of eight subclone replicates). These haplotypes had substitutions at 19 different positions, only one of which was a transversion, and no indels. All of the substitutions were at the third codon position and none of them resulted in a stop codon.

Table 2. Sampling localities and frequencies for each haplotype

Haplotype	GenBank accession number	Locality																				
		075	076	077	078	079	080	081	082	083	085	086	100	104	105	110	111	112	113	Ger.A	102*	103*	108*
D1	AY771280	5	7	4	7	4	5	5	2														
D2	AY771303			1																			
D3	AY771304					2																	
D4	AY771305									4	6	7											
D6	AY771281												5	1	6					1			
D7	AY771307												2										
D8	AY771308													4									
D9	AY771309														1								
D10	AY771310															5							
D11	AY771311															5	2	5	3				
D12	AY771282																2						
D13	AY771312																2						
D14	AY771313																2		7				
D15	AY771314																2						
D16	AY771315																2						
D17	AY771316																	5					
D18	AY771293																				9	7	
D19	AY771317																				6	7	
D20	AY771318																					1	
D21	AY771294																						14
D22	AY771319																						1

Localities are described in Table 1.
*Sites containing populations of *Theodoxus prevostianus*.

These features mean that these haplotypes are still functional and do not possess any signatures of nuclear gene capture (Zhang & Hewitt, 1996; Thalmann *et al*., 2004; Pons & Vogler, 2005). Furthermore, when these haplotypes are included in a phylogenetic analysis, the branching patterns of the two clades into which they fall are highly correlated (see below and Fig. 2). These two clades also each give rise to populations that do not display any CO I polymorphism, indicating that both clades include mitochondrial CO I genes. Furthermore, because some individuals contain multiple haplotypes that are assignable to each of these two clades (e.g. one individual from site 111 possesses haplotypes D11, D13, D14, D15, and D16), it is not possible to declare one of the two haplotype lineages as being of nuclear origin because single individuals should only have multiple haplotypes from one clade. Therefore, haplotypes from both clades are most likely to be of mito-

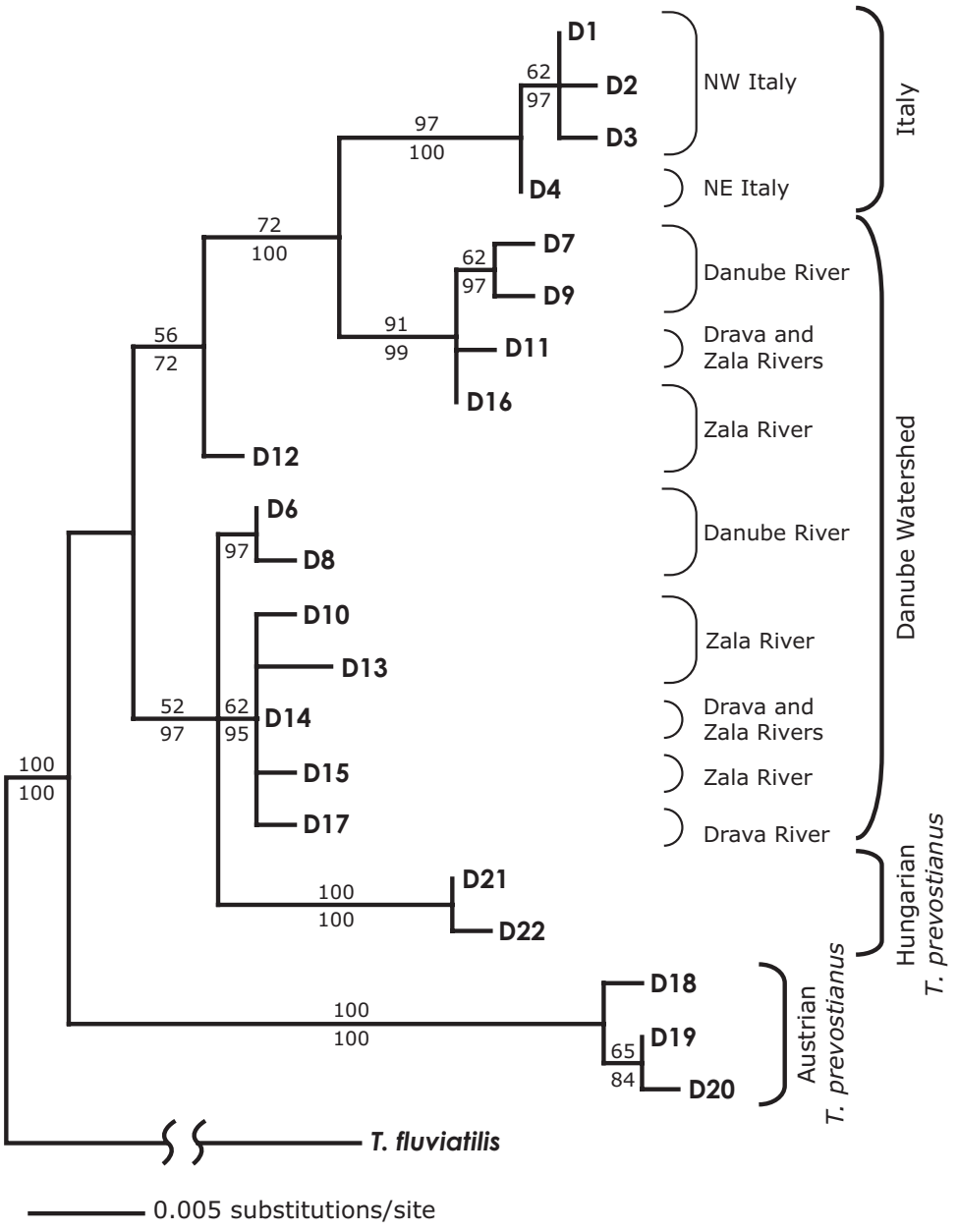

Figure 2. Maximum likelihood tree of all unique haplotypes for *Theodoxus danubialis* and *Theodoxus prevostianus*. The tree recovered by parsimony is identical. Haplotypes are as described in Table 2. Bootstrap values higher than 50% are above the branch, Bayesian posterior probabilities are below the branch. Regions describe the areas from which haplotypes were sampled.

chondrial origin. This was unexpected based on the presumed maternal inheritance and generational bottlenecks of mitochondria. However, mitochondrial DNA sequence polymorphisms are known in gastropods (Davison, 2000; Terrett, Miles & Thomas, 1994) and bivalves (Zouros *et al*., 1994; Curole & Kocher, 2002), and may represent a unique mode of mitochondrial inheritance (Zouros *et al*., 1994).

Haplotypes each consisted of 600 bp. Of 47 variable sites, 33 were parsimony-informative, and the estimated transition/transversion ratio was 7.8. The ML analysis produced a single tree with a score of 1263.2055 (Fig. 2). The MP analysis produced a single most parsimonious tree that was identical to the ML tree, 55 steps in length, with a consistency index (CI) of 0.891 and a retention index (RI) of 0.995. ML bootstrap percentages and Bayesian posterior probabilities are shown in Figure 2. The largest pairwise difference between haplotypes is 4.0%. Most haplotypes are separated by only one or two nucleotide substitutions and the mean ± SD pairwise distance is 2.1 ± 1.1%. This degree of divergence is only 1.6 ± 0.8% when *T. prevostianus* specimens are ignored.

The populations assigned to *T. prevostianus* do not form a monophyletic group. It is unclear whether these individuals represent a paraphyletic unit or a polyphyletic one because CO I was unable to resolve their relationships within the *T. danubialis*/*T. prevostianus* group. The two populations from Austria form a single, well-supported clade (all metrics above 99%) containing two common haplotypes, distributed evenly between both populations, and a third haplotype (D20) which is one nucleotide substitution different from haplotype D19. This *T. prevostianus* clade appears to be basal to the rest of the *T. danubialis* clade (Fig. 2) and is distinct by 3.0% sequence divergence. The *T. prevostianus* samples from Hungary (locality 108) form a monophyletic group (all metrics 100%) and are related to *T. danubialis* from the Danube and its tributaries (Fig. 2). They diverge by approximately 1.2% sequence divergence. Fourteen of the Hungarian *T. prevostianus* individuals had the same haplotype and one individual had a haplotype (D22) that differed by a single substitution. A monophyletic *T. prevostianus* is not supported by any characters when a constraint tree is enforced. Estimation of the posterior probability of a monophyletic *T. prevostianus* can be estimated by the proportion of all trees from the Bayesian analysis consistent with such a pattern (Buckley, 2002). This posterior probability is only 0.0065, indicating that monophyly for *T. prevostianus* can be rejected.

Two clades present within the western Danubian province received support (Bayesian posterior probabilities of 97% and 99%), and were each represented in all populations in all three river drainages sampled: the upper Danube River drainage, the Zala River drainage, and the Drava River drainage. One of these two clades includes the Hungarian *T. prevostianus* population. The other clade found in the Pannonian basin is the sister clade to the Italian samples (100% posterior probability). One haplotype from the Zala River in Hungary, D12, was found in an intermediate position between the two primary *T. danubialis* clades. The Italian samples included two common haplotypes, D1 and D4, and two others, D2 and D3, which were found in only one and two individuals, respectively. Haplotype D4 is found in north-eastern Italian tributaries of the Adriatic (sites 083, 084, 085, and 086). The other three haplotypes are found mainly in the Po River drainage, west of the distribution of haplotype D4. Only two populations of the north-west Italian clade, 081 and 082, are not in tributaries of the Po River, but their mouths are very near to the mouth of the Po River just south of the Venetian Lagoon. One of the two non-Po populations (081) is in a canal that is tributary to the Bacchiglione River, from which population 083 was sampled and which contains the basal Italian haplotype D4. There is substantial statistical support (97% bootstrap, 100% posterior probability) for a clade containing all Italian samples, which is physically separated from their closest relatives in the Danube watershed by the Dinaric Alps. Additionally, there is some statistical support for the conclusion that the three western Italian haplotypes are derived from the eastern Italian haplotype D4 (62% bootstrap, 97% posterior probability).

Several of the populations have haplotypes that belong to the two primary Danubian clades. Several individuals, those with polymorphic genomes, possess haplotypes that are in these two distinct clades. The branching patterns of these two clades are highly similar, with haplotypes from the Drava and Zala Rivers more closely related than either is to haplotypes from the Danube.

The population from the Wiener Neustadter Kanal (site 104) is almost entirely different from its nearest relatives in the Danube River. Four of the five sampled individuals share a haplotype (D8) that is one mutational step different from the most common haplotype of the Danube River (haplotype D6; sampled at two sites in Austria and one in Bavaria). The fifth individual possesses this more common Danubian haplotype. Within the three other upstream Danube populations (sites 100, 105, and Ger.A), the majority of individuals possessed this dominant haplotype, including the single individual sequenced from far upstream in Bavaria (site Ger.A). This is consistent with the evolution of a derived haplotype (D8) in the Wiener Neustadter Kanal following the physical separation of this canal from the Danube River.

Nested clade phylogeographical analysis

The haplotype network and the series of nested clades are described in Figure 3. The nesting procedure (Templeton & Sing, 1993) resulted in 30 nested clades, eight of which could reject the null hypothesis of no phylogeographical structure. A summary of the results for those clades with significant geographical association or dispersion is presented in Table 3.

Of the eight clades that showed some geographical structure, four were inferred to result from some type of fragmentation, three involved range expansion, and the other may result from some combination of these processes. Two of the clades showing allopatry involve the distant Italian populations. One, clade 2-1, appears to indicate allopatry between western and eastern Italian populations, concordant with the interpretation from the phylogenetic analysis. The other allopatric clade, 3-1, implies that the Dinaric Alps separating Italy from the Pannonian basin has led to an allopatric distribution. Within the Danube watershed, clade 1-3 has a distribution that is inferred to result from allopatry, wherein the population in the Wiener Neustadt Kanal is genetically and geographically distinct from nearby Danube River populations.

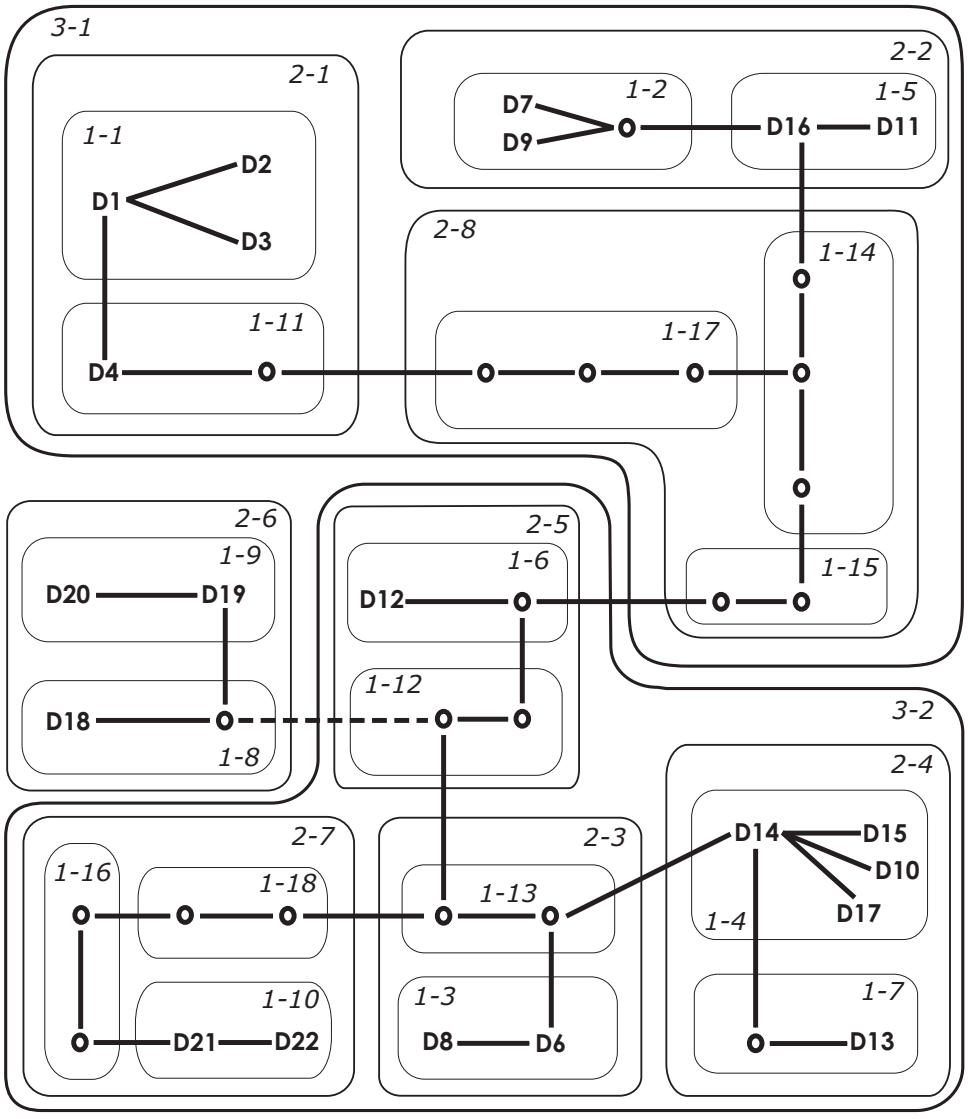

Figure 3. Haplotype network for *Theodoxus danubialis* and *Theodoxus prevostianus*. All nesting levels are shown. '*D#*' indicates a unique haplotype (for the distribution of each haplotype, see Table 2). ○, inferred intermediate haplotypes. The dashed line between clade 2-6 (Austrian *T. prevostianus*) and the remainder of the network is the relationship found by the phylogenetic analyses (Fig. 2).

Table 3. Results of the nested clade phylogeographical analysis of *Theodoxus danubialis*/*Theodoxus prevostianus*

Clade	Nested haplotypes/clades	D_c (km)	D_n (km)	Inference chain
1-3				1-2-3-4-9-No: AF
	D6	165.1†	158.2	
	D8	0.0*	89.8	
	I-T	165.1†	68.3	
1-4				1-2-3-5-6: REC/RGF
	D10	0.0*	54.7†	
	D14	30.1*	44.8	
	D15	0.0	54.7†	
	D17	0.0*	31.6	
	I-T	30.1†	–0.3	
2-1				1-19-No: AF
	1-1	50.7*	67.1*	
	1-11	51.3*	121.6†	
	I-T	0.6	54.5†	
2-2				1-19-20-2-11-12-No: CRE
	1-2	41.1	135.7†	
	1-5	45.2*	56.5*	
	I-T	4.1	–79.2*	
3-1				1-19-No: AF
	2-1	80.0*	102.7*	
	2-2	73.0*	389.8†	
	2-8	0.0	0.0	
	I-T	0.0	0.0	
3-2				1-2-3-5-6: REC/RGF
	2-3	142.9	139.5	
	2-4	46.0*	118.9	
	2-5	0.0	112.9	
	2-7	0.0*	314.4†	
	I-T	100.0†	–59.4	
4-1				1-2-11-12-13-14-Yes: LDC (+ F/REC)
	3–1	142.9*	210.7*	
	3–2	157.5*	325.3†	
	I-T	14.6	114.5†	
Total cladogram				1-19-No: AF
	4-1	247.5	252.9	
	2-6	7.6*	256.1	

Only those clades which could reject the null hypothesis of no phylogeographical structure are shown.
*Distances that are significantly ($P < 0.05$) smaller than expected.
†Distances that are significantly ($P < 0.05$) larger than expected.
D_c, geographical spread within a clade; D_n, geographical spread between nested clades; I-T, average distance between interior and tip nodes in a given clade; AF, allopatric fragmentation; F, fragmentation; REC, range expansion/colonization; RGF, restricted dispersal/gene flow; CRE, contiguous range expansion; LDC, long-distance colonization.

Of the three clades whose distribution is inferred to result from range expansion, two of them (clades 1-4 and 2-2) are the primary lineages present in the eastern Danube watershed, indicating that the relationships of populations in the Danube, Drava, and Zala rivers has resulted in large part from contiguous and/or long-distance range expansion. These populations all have concordant separation within two shared clades, the polymorphic individuals generating replicate nested clades with similar NCPA statistics. Because the nesting procedure arbitrarily groups clades with equivalent genetic distances (Templeton & Sing, 1993), these two nesting clades are not at the same nesting level, although they both represent all Pannonian haplotypes in each of the two CO I clades. The third clade displaying range expansion is the nesting clade 3-2, which includes nested clade 1-4 as well as the Hungarian *T. prevostianus* populations,

indicating that range expansion is a common pattern for these snails in the Pannonian basin.

The Danube of south-eastern Hungary and Romania, which is where individuals from these three rivers would have to intermingle, was not sampled and may not contain many, if any, remaining populations of *T. danubialis* (Frank, 1982). Between the populations of the western Danube watershed, the NCPA indicates that fragmentation *per se* was not a factor in developing phylogeographical structure (Table 3), although it may have played a role. Inference for the phylogeographical pattern of this clade (4-1) by the NCPA is confounded by the inclusion of the distant Italian populations and the endemic Hungarian *T. prevostianus* population.

Within the samples assigned to *T. prevostianus*, phylogeographical structure was inferred as predicted. The clade including the two Austrian populations (2-6) could not be joined to the rest of the network under the 95% confidence interval of the statistical parsimony analysis. The relationship of this clade to the rest of the *T. prevostianus*/*T. danubialis* group was inferred from the ML and MP trees. The NCPA infers phylogeographical structure between this clade and the rest of the network but does not find any phylogeographical structure within the clade containing the two Austrian populations. As expected, given their restricted distribution and adherence to thermal springs, the NCPA infers allopatric fragmentation as the most likely mechanism by which these populations have differentiated. The Hungarian *T. prevostianus* population is separated from the rest of the haplotype network by six mutational steps and shows a strong geographical association according to the results of GeoDis. Either range expansion/colonization or restricted dispersal/gene flow is inferred to be the phylogeographical structuring mechanism for the nesting clade 3-2, which includes the Hungarian *T. prevostianus* and its closest relatives. This is compatible with the fact that no populations of *T. danubialis* are known from within 200 km of this population (site 108).

Genetic diversity and mismatch distribution

The haplotype diversity for the *T. danubialis*/*T. prevostianus* clade was 0.897 ± 0.012 and the nucleotide diversity was 0.021 ± 0.010. These values represent relatively low genetic diversity for neritid gastropods but are slightly higher than those found for *T. fluviatilis* (Bunje, 2005). The measurement of pairwise differences between sampled haplotypes revealed a Poisson-like, unimodal distribution for the Italian specimens (Fig. 4). This distribution did not differ ($P < 0.001$) from the expected distribution under a model of rapid population expansion (Rogers & Harpending, 1992). The analysis of the entire *T. danubialis*/*T. prevostianus* clade did not reveal a Poisson-like distribution of pairwise mismatch differences ($P = 0.113$; Fig. 4). Only one other clade, the Austrian *T. prevostianus* populations, could not be distinguished from the simulated distribution ($P < 0.05$), but it was distinctly bimodal (not shown). No other subclades of *T. danubialis*/*T. prevostianus* showed a unimodal distribution and all others were distinguishable from their respective simulated distributions ($P > 0.1$), including each of the two Pannonian basin clades, the Hungarian *T. prevostianus* clade, the Pannonian clade including its Italian descendants, and the Pannonian clade including the related Hungarian *T. prevostianus*. Taken together, these results are consistent with the hypothesis that only populations in

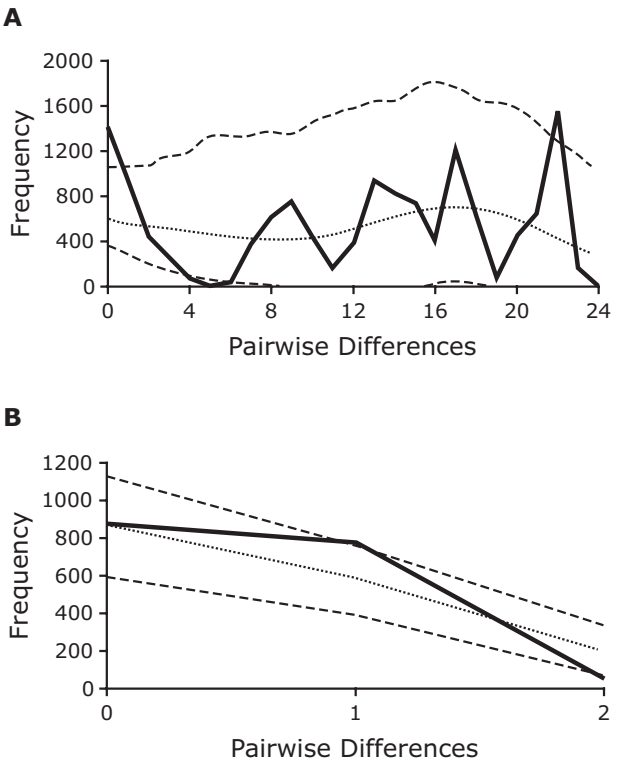

Figure 4. Results of the pairwise mismatch distribution analysis for the entire *Theodoxus danubialis*/*Theodoxus prevostianus* clade (A) and the samples from Italy (B). The observed data (solid line) for Italian samples (B) are not distinguishable from the simulated (dotted line) Poisson-like distribution ($P < 0.001$). All other clades present in the phylogenetic analyses (the two Pannonian basin clades, the Hungarian *T. prevostianus* clade, the Pannonian clade including its Italian descendants, and the Pannonian clade including the related Hungarian *T. prevostianus*), as well as the entire clade (A), deviated from the unimodal Poisson-like distribution expected under a model of exponential population growth (Slatkin & Hudson, 1991).

Italy have undergone recent population growth (Slatkin & Hudson, 1991).

DISCUSSION

Intraindividual polymorphisms

The discovery of multiple haplotypes within many of the sampled individuals is noteworthy. Methods generating mitochondrial sequence polymorphism within individuals include 'ghost genes' in the nucleus, biparental inheritance, large numbers of mitochondria being sequestered during gametogenesis, and nonneutral sequence evolution in heteroplasmic lineages (Zhang & Hewitt, 1996). As noted above, heteroplasmy is known in mollusks (Terrett *et al*., 1994; Zouros *et al*., 1994; Davison, 2000; Curole & Kocher, 2002) and may represent the best explanation for the intraindividual polymorphism found in *T. danubialis*. If some of the subcloned haplotypes are not mitochondrial, however, then the most likely origin of this intraindividual polymorphism is the presence of a nuclear mitochondrial pseudogene (Numt) within the nucleus (Bensasson *et al*., 2001; Zhang & Hewitt, 1996). Infrequent events of gene transfer between the mitochondrial and nuclear genome followed by subsequent divergence of the nuclear gene, freed from the constraints of stabilizing selection, can lead to Numts (Bensasson *et al*., 2001).

The polymorphisms within *T. danubialis* CO I do not show any of the typical signs of nuclear gene capture, such as sequence length variation, indels, transversion bias, or stop codons (Zhang & Hewitt, 1996; Thalmann *et al*., 2004; Pons & Vogler, 2005). Furthermore, mollusks do not appear to possess as many Numts as other metazoan groups (Bensasson *et al*., 2001) and the sequencing of another mitochondrial gene, 16S rRNA, from the same specimens and other species of *Theodoxus* produced no signs of polymorphism (Bunje, 2004). The phylogenetic analysis of all haplotypes also provides evidence against the presence of a Numt. Each Pannonian clade has given rise to populations that display no polymorphism (Fig. 2), indicating that both clades are comprised of functional copies of the CO I gene, making it highly unlikely that either clade is comprised of nuclear sequences. Additionally, single individuals were identified that possess more than one haplotype from each of the Pannonian clades. If either clade was comprised of Numts, then the other clade should be present as a single haplotype in each individual. Taken together, this evidence points to heteroplasmy as the most likely candidate for explanation of intraindividual polymorphism in *T. danubialis*.

Some populations have lost one of the mitochondrial lineages, probably as a result of bottleneck events at gamete formation, producing the nonheteroplasmic clades in Italy and the western Danube River. Given that heteroplasmic populations normally show preference for one mitochondrial type as a result of lineage sorting (Casane *et al*., 1997), this scenario is plausible. Regardless of the origin of these polymorphisms, the phylogeographical interpretations presented in the present study are valid because of the nature in which haplotypes form gene trees, whether they be nuclear or mitochondrial (Zischler, Geisert & Castresana, 1998; Bensasson *et al*., 2001; Pons & Vogler, 2005).

Fluvial range expansion and localized allopatry in the Danube watershed

Both the phylogenetic analysis of haplotypes and the nested clade phylogeographical analysis revealed significant interpopulational structure that is mainly associated with watersheds within the *T. danubialis*/*T. prevostianus* lineage. As expected from their restricted ranges and unique morphologies, the populations of *T. prevostianus* are genetically distinct from the rest of the *T. danubialis* clade. The implications of this distinctiveness and its multiple independent origins are discussed below. For the rest of the clade, processes of range expansion and fragmentation are apparent.

Within the western Danubian watershed, the three major river drainages contain two distinct clades demonstrating complementary phylogeographical structure (Fig. 2). The haplotypes from the Zala and Drava Rivers are more closely related to each other whereas the haplotypes from the upper Danube are distinct in both instances. Having two examples of similar structure spread across the same geographical area, albeit facilitated by the migration of single individuals and therefore non-independent, lends strong support to the general phylogeographical patterns found. Additionally, finding two replicates of the same phylogenetic structure implies that the phylogeographical processes producing this structure have occurred recently enough to be preserved in the gene trees of these individuals without being obscured by either gene flow or lineage sorting (Maddison, 1997).

The general pattern found (i.e. that of haplotypes primarily restricted to three separate rivers) is indicative of significant phylogeographical structure. Only two haplotypes (D11 and D14) are found in more than one river, and each is found in both the Drava and Zala Rivers and comes from the two distinct clades. Given that these geographical patterns occur twice in parallel, a reasonable explanation would be that the migration of polymorphic individuals upstream from a downstream (lower Danube) source population has occurred with the concomitant divergence of lineages within the upstream tributaries (Fig. 5). The NCPA generally agrees with this interpretation insofar as it

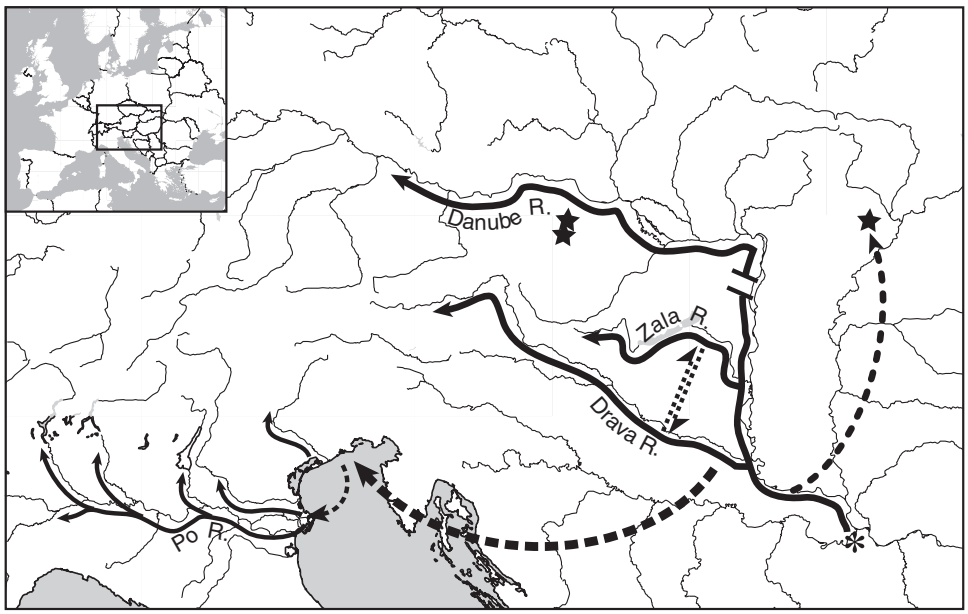

Figure 5. Hypothetical migratory history of *Theodoxus danubialis*. The thickness of the lines is relative to the hypothesized age of the event, with thicker lines representing older events. From a lower Danube River source population (*), two haplotype lineages moved upstream, colonizing the Drava, Zala, and upper Danube Rivers. Possible gene flow between populations in the Drava and Zala Rivers is indicated by the double dashed line, although shared haplotypes may simply result from shared ancestry. More recently, colonizing individuals reached north-eastern Italy, either from the Danube basin itself or from related populations in the eastern Adriatic Sea. These colonizers founded populations westward, reaching the Po River and its tributaries. Populations of *Theodoxus prevostianus* (★) are of unknown origins and represent ancient lineages, perhaps dating to the Miocene (Lueger, 1979). The two populations in Austria are probably older than the hypothesized movements shown here. The Hungarian *T. prevostianus* is related to the Danubian *T. danubialis*, but its precise origin is unknown.

infers the phylogeographical structure of clades 2-2 and 3-2 as being due to range expansion. This hypothesis requires that the ancestral haplotypes for all three regions existed in a common area (i.e. the Danube east of the confluence with the Drava River). The upper Danube populations are clearly distinct and always form monophyletic groups to the exclusion of the Zala and Drava river populations, which are ambiguously resolved in these analyses. Gene flow may still be occurring between tributaries such as the Zala and Drava rivers, although the ambiguous relationships between these rivers may also result from incomplete lineage sorting.

Outside of the *T. prevostianus* populations, which are discussed below, allopatry is apparent in at least one area of the Danube, in the population from the Wiener Neustadter Kanal (site 104). This canal was once connected to the Danube River near Vienna but has subsequently become isolated. The haplotypes now present in the canal are distinct, with one individual possessing the ancestral haplotype from the Danube and the other four sharing a unique derived haplotype. This pattern is consistent with drift within this isolated population under neutral mitochondrial evolution.

The patterns observed within the Danube watershed are similar to those found for several fish species (Economidis & Banarescu, 1991; Durand *et al*., 1999b; Bernatchez, 2001; Salzburger *et al*., 2003). The Danube River is considered to be an important source area for the colonization of nearby watersheds and mountainous regions affected by glaciation during the Pleistocene (Durand *et al*., 1999a; Economidis & Banarescu, 1991). A similar pattern is supported for *T. danubialis*, wherein the likely most recent common ancestor of sampled populations is in the lower Danube River of Hungary and Romania (Fig. 5). Further sampling of the remaining populations from the Danube River and its eastern tributaries is needed to confirm and clarify this prediction.

Allopatry and range expansion in Italy

The importance of the Danube watershed in contributing to the fauna of other European drainages (Economidis & Banarescu, 1991; Durand *et al*., 1999a; Perdices *et al*., 2003) is demonstrated by the history of Italian populations of *T. danubialis*. All Italian populations of *T. danubialis* are closely related and repre-

sent a mitochondrial lineage in a derived position within the *T. danubialis*/*T. prevostianus* clade. One of the two Pannonian clades has given rise to all populations in Italy (Fig. 2). The NCPA hypothesizes both fragmentation and range expansion as explanations for this pattern, interpretations that are consistent with the phylogeny and geographical distribution of this species. Italy is separated from the Danube watershed by the mountains of Slovenia, where the Dinaric Alps and Alps converge. This barrier has apparently helped separate the populations, creating distinct phylogenetic units on either side of the Alps (Fig. 5).

The Italian clade is separated by six nucleotide substitutions from its closest relative. However, within the Italian clade, there are only four unique haplotypes, and they are at most one substitution different from their respective ancestors. This, in addition to the pairwise mismatch distribution (Fig. 4), is evidence of recent population growth following a significant fragmentary or founder event. Furthermore, the pattern observed places the ancestral haplotypes in the north-east of Italy, meaning that all haplotypes to the west (in the Veneto-Padano basin) are descendants of this. All but two specimens from the Veneto-Padano basin belong to a single haplotype, supporting the conclusion that this species has recently expanded its range westward.

Bodon & Giovanelli (1995) hypothesized that the Italian populations of the Veneto-Padano basin were the result of recent, perhaps human-mediated, colonization. Unfortunately, I cannot confirm the age of the inferred range expansion. However, in the closely related species *T. fluviatilis*, most post-Pleistocene populations of northern Europe still possess the original, colonizing haplotype (Bunje, 2005). Similarly, populations of the Veneto-Padano basin could thus be at least as old as the last glaciation.

The branch separating the Italian clade from Pannonian populations is long and well supported compared to the rest of the tree. The phylogenetic tree indicates that Italian populations are members of one of the two primary Pannonian haplotype lineages. This implies that at least one of these two Pannonian lineages has a distribution greater than the western Danube watershed and may be seeding new populations in other waters of Europe. Consequently, it appears that range expansion is an ongoing process in *T. danubialis* and an important aspect in the continued assembly of European freshwater faunas.

Multiple origins and parallelism in *T. prevostianus*

The calcareous thermal springs in which *T. prevostianus* lives are all in areas that were dry highlands when the Pannonian basin was filled with Lake (or Sea) Pannon, and the springs have subsequently remained generally unconnected to major drainage systems (Pintér *et al*., 1979). In the springs that do have streams flowing into larger river systems, *T. prevostianus* is only found at most a few hundred meters downstream of the spring (Piringer, 2002). In such restricted habitats, these small, inky-black spring snails can be found in great abundance. *T. prevostianus* has also been reported from Romania and northern Greece (AQEM project, http://www.aqem.de), but I was unable to confirm this and it is difficult to assess the likelihood that these snails represent related populations.

This phylogeographical analysis supports the hypothesis that *T. prevostianus* is a nonmonophyletic taxon. The ambiguity in the branching pattern found in both the parsimony and likelihood analyses makes it difficult to declare where Hungarian *T. prevostianus* fits in the tree. Other genes may change the placement to be at the base of the clade containing all *T. danubialis*, thus indicating that *T. prevostianus* is paraphyletic, and not polyphyletic as indicated by this analysis. A polyphyletic interpretation means that the Austrian and Hungarian populations do not form a single lineage, but rather they are the result of two independent origins of morphologically and ecologically similar lineages. These two lineages are distinct genetically, both showing high degrees of divergence from their nearest relatives within *T. danubialis* (1.2% for Hungarian, 2.8% for Austrian). This amount of divergence is relatively large compared to the average pairwise distance in the closely related *T. fluviatilis*, which is 1.5% at most (Bunje, 2005), indicating that these represent independently evolving lineages within *Theodoxus*.

The contradiction between monophyletic species definitions and lineage independence is a particular conundrum for understanding species formation. Not until enough time has passed for lineages to sort into independent phylogenetic units can these 'species' be considered as separate under most concepts (de Queiroz, 1998). However, these sorting processes are likely to be extremely long in species with restricted endemic lineages nested within a more widely distributed group (Strecker *et al*., 1996). It is likely that species or clades that inhabit geologically complex regions, such as freshwater species in continents or terrestrial species on continental islands, will show a high frequency of polyphyly within interbreeding or cohesive species (Melnick *et al*., 1993; Brown *et al*., 1994; Rieseberg & Brouillet, 1994). The area of eastern Europe that this clade inhabits is geologically complex and has a history of frequent glaciation, river capture, volcanism, variable sedimentation, and tectonics that have led to a constantly changing set of suitable habitats (Bernatchez, 2001; Salzburger *et al*.,

2003). The origin of multiple, independent lineages that are ecologically and morphologically indistinguishable is therefore not unexpected, although it is striking.

In addition to what this clade can tell us about parallelism and species formation, it is important to recognize the uniqueness of these snails in the context of conservation. With low genetic diversity in independently evolving populations, the two *T. prevostianus* lineages are susceptible to extinction. Unfortunately, most of their habitat has been destroyed, making it difficult to evaluate competing hypotheses for their interesting evolutionary history. The two known populations in Austria are now protected and celebrated. Out of seven populations known from Hungary in the 1950s (Pintér *et al*., 1979), only one remains. All of the other populations have been altered so significantly by humans, through drying of springs, chlorination for baths, and pollution, that *T. prevostianus* no longer inhabits these springs. The single population remaining in Hungary is also distinct genetically. It is unfortunate that we have lost the other populations and their potentially unique diversity. Perhaps other populations remain in areas surrounding the southern and eastern borders of the Pannonian basin; these populations of *T. prevostianus* should illuminate the processes leading to parallelism and lineage divergence in these remarkable spring snails.

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