

Evidence of a genetically distinct population of Vrljika softmouth trout *Salmo obtusirostris* Heckel evolved by vicariance

A. SNOJ*, I. BOGUT†, S. SUŠNIK*‡

*University of Ljubljana, Department of Animal Science, Groblje 3, SI-1230 Domžale, Slovenia and †University of Osijek, Agricultural Faculty, Trg sv. Trojstva 3, 31000 Osijek, Croatia

(Received 30 July 2007, Accepted 21 December 2007)

Mitochondrial DNA haplotypes (control region, partial cytochrome *b* and ATPase6 genes) indicate a sister relationship between Vrljika and Neretva softmouth (Adriatic) trout *Salmo obtusirostris*. This relationship was supported by a tree of individuals based on microsatellite results [allele sharing distances (D_{AS})], which revealed three distinctive clusters, corresponding to Jadro softmouth, Neretva brown trout *Salmo trutta* and Neretva softmouth trout. Within the latter taxon, Vrljika trout are clearly separated from other trout. The genetic results contradict the synonymy of Jadro with Vrljika softmouth trout, as recently proposed in the Red Book of Freshwater Fish in Croatia. Vrljika softmouth trout appear to have originated from a vicariance that split a common ancestor into large (Neretva) and small (Vrljika) fragmented populations 135 000–270 000 years ago. Vrljika softmouth trout can be distinguished by an array of derived phenotypic and molecular character states. For conservation, this population should be recognized formally at the same taxonomic level as the other geographically separated populations of softmouth trout.

© 2008 The Authors

Journal compilation © 2008 The Fisheries Society of the British Isles

Key words: Balkan Peninsula; conservation; freshwater fish; habitat fragmentation; molecular classification; River Vrljika.

INTRODUCTION

Knowledge of spatial and temporal intraspecific genetic variation is a key element for understanding the evolutionary history of a species. Current patterns of genetic structure are likely to be the result of complex interactions among many processes, such as vicariance, habitat fragmentation, dispersion, gene flow and founder effects, among others (Mayr, 1942, 1963; Branco *et al.*, 2002). Of these processes, habitat fragmentation is an important factor that can lead to population differentiation. Isolated populations may start to differentiate through genetic drift (Avice, 2000), perhaps accompanied by novel selection pressures (Seehausen, 2004). In the case of a small fragmented population, founder and inbreeding effects generated by bottlenecks may further enhance a new genetic identity (Page & Holmes, 2001).

‡Author to whom correspondence should be addressed. Tel.: +386 1 7217 912; fax: +386 1 7241 005; email: simona.susnik@bfro.uni-lj.si

Karstic terrain, with a typical hydrological system containing small discontinuous surface streams, connected only by subterranean passages represents a good example of habitat fragmentation. Hydrographic structuring of water lice (*Asellus aquaticus* L.) populations has been recently reported for surface and subterranean waters of the Dinaric karst of the north-western Balkans (Verovnik *et al.*, 2004), while several fish populations also inhabit similar fragmented karstic habitats (Bogutskaya & Zupančič, 2003; Freyhof *et al.*, 2006), including the softmouth (Adriatic) trout, *Salmo obtusirostris* Heckel (Schöffmann, 2003). No literature exists on either the phylogenetic relationships of such fish populations or their times and patterns of colonization or fragmentation. This study focuses on *S. obtusirostris*, which is endemic to highly restricted areas of the peri-Adriatic karstic region of the western Balkan Peninsula.

Populations of softmouth trout naturally inhabit four rivers (Krka, Jadro, Neretva and Zeta) that drain superficially into the Adriatic Sea, as well as the River Vrljika, which rises in the north-western part of the Imotsko Polje in the Dinaric karst of south-western Croatia and drains *via* subterranean passages into the Neretva basin (Fig. 1). Each of these geographically separated populations is characterized by a phenotypically distinct form (www.balkan-trout.com), with some authors classifying them as sub-species (Mrakovčić *et al.*, 2006). Until now, only softmouth trout in the rivers Neretva (*Salmo obtusirostris oxyrhynchus* Steindachner), Jadro (*Salmo obtusirostris salonitana* Karaman) and Zeta (*Salmo obtusirostris zetensis* Hadžišće) have been given scientific attention (Snoj *et al.*, 2002; Sušnik *et al.*, 2007a, b).



FIG. 1. Geographic locations of *Salmo obtusirostris* populations sampled in the Jadro, Vrljika and Neretva rivers. Sampling locations are indicated with arrows.

Phylogenetic analysis of a combined data set of mitochondrial (mt) and nuclear DNA indicates that Neretva softmouth trout are distinct from brown trout (*Salmo trutta* L.) and supports its inclusion in the genus *Salmo*, but as a separate species (Snoj *et al.*, 2002). In contrast, Jadro softmouth trout are fixed for a brown trout mtDNA haplotype of the Adriatic lineage (Sušnik *et al.*, 2007b), while nuclear and morphological characteristics are typical of Neretva softmouth trout, indicating an mtDNA capture event.

Vrljika trout, which are a distinctive golden yellow, have been neglected since Heckel's original description in 1851. Moreover, this taxon was considered to be extinct by local ichthyologists (Mrakovčić & Mišetić, 1990). However, Schöffmann (2004) recently reported field observations of softmouth trout in the River Vrljika. According to local residents, softmouth trout are the only salmonid known to have inhabited this river, and this is the current situation (pers. obs.). In the Red Book of Freshwater Fish in Croatia (Mrakovčić *et al.*, 2006), Vrljika softmouth trout have been equated to the Jadro softmouth trout with the statement that 'the distribution of *Salmothymus obtusirostris salonitana* (i.e. Jadro softmouth trout) is confined to the rivers Jadro, Žrnovnica and Vrljika'. No details to support this view were given.

The watershed of the River Vrljika lies between the Jadro and Neretva rivers. After a few kilometres above ground, the river disappears to reappear later as the River Trebižat, which flows into the River Neretva. The physical connection of the river systems inhabited by the Neretva and Vrljika softmouth trout and high mountain range of Mosor and Biokovo (Fig. 1), separating the River Jadro from the western part of the Neretva basin, indicate that Vrljika softmouth trout might have originated from Neretva softmouth trout. Thus, the view that a close relationship exists between Jadro and Vrljika softmouth trout is questionable. It is therefore important for conservation to determine accurately the taxonomic position of Vrljika softmouth trout in order to be able to implement appropriate management strategies.

To address these issues, the phylogeny of Vrljika softmouth trout was estimated with mtDNA and nuclear DNA markers. By including paleogeographic information, the aim was also to determine the time of divergence of the Vrljika population from its nearest relatives and to propose a possible situation for the colonization of this river.

MATERIALS AND METHODS

Fin clips from 23 individual softmouth trout from the River Vrljika (Fig. 1) were analysed along with samples collected in the lower part of the Neretva ($n = 33$) and Jadro ($n = 10$) rivers. Published data for Jadro softmouth trout and Neretva brown trout were included for comparison (Sušnik *et al.*, 2007a). Whole genomic DNA was isolated from the fin tissue using a high salt extraction technique (Miller *et al.*, 1988).

SEQUENCE ANALYSIS

Comparative nucleotide analysis of three mtDNA regions [control region (CR), part of the cytochrome *b* and ATPase6 genes] and two genomic DNA regions [part of the lactate dehydrogenase *LDH-C1** gene and internal transcribed spacer (ITS1) region] was undertaken for the samples collected from the three rivers (Table 1). Polymerase

TABLE I. Number of individuals from the Vrljika, Neretva and Jadro rivers sequenced at particular DNA regions and GenBank accession numbers

Haplotype	<i>n</i>	Sequenced regions				
		mtDNA			Genomic DNA	
		Control region	<i>Cyt b</i>	ATPase6	ITS	<i>LDH-CI</i> *
Vrljika	23	23 (EF469832)	5 (AF488534)	5 (EF469834)	5 (AY260509)	5 (AF488540)
Neretva	33		5 (AF488534)	5 (EF469835)	5 (AY260509)	5 (AF488540)
Soxy 1		32 (AF488535)				
Soxy 2		1 (EF469833)				
Jadro	10	10 (AY653218)*	5 (AY653214)*	5 (EF469836)	5 (AY260509)*	5 (AF488540)*

*Data from Sušnik *et al.* (2007b); ITS, internal transcribed spacer.

chain reaction (PCR) amplification of a *c.* 2400 base pairs (bp) mtDNA fragment composed of the cytochrome *b* gene and CR was performed using primers HN20 (Bernatchez & Danzmann, 1993) and C-Glu (Cronin *et al.*, 1993). In addition, part of the ATPase6 gene (650 bp) was amplified using the L8558 and H9208 primers described in Giuffra *et al.* (1994). PCR conditions were as follows: each 25 µl reaction contained 1 µM of each primer, 0.2 mM dNTP, 1.5 mM MgCl₂, 1 × PCR buffer, 1 U *Taq* polymerase (AB Applied Biosystems, Foster City, CA, USA) and 100 ng genomic DNA; the cycle variables included initial DNA denaturation (95° C, 3 min) and 30 successive cycles of strand denaturation (94° C, 45 s), primer annealing (53° C, 45 s) and DNA extension (72° C, 2 min).

Primers Ldhxon3F and Ldhxon4R were used for amplification of a *c.* 440 bp fragment of *LDH-CI**, as described in McMeel *et al.* (2001), while primers KP2 and 5.8S were applied for amplification of 650 bp ITS1 region (Phillips *et al.*, 2000; Presa *et al.*, 2002).

Amplified DNA fragments were separated in a 1.5% agarose gel and isolated using a QIAEX II Gel Extraction kit (QIAGEN, Hilden, Germany). Approximately 100 ng of the purified PCR product was used in cycle sequencing reactions, following ABI PRISM BigDye Terminator protocols (AB Applied Biosystems). The sequencing primers were 28RIBa (Snoj *et al.*, 2000) for the 5'-end and HN20 for the 3'-end of CR, C-Glu for the 5'-end of cytochrome *b* and H9208 for ATPase6, and Ldhxon4R and 5.8S for the sequencing of *LDH-CI** and the ITS1 region, respectively. The amplified, fluorescently labelled and terminated DNA was salt-precipitated and visualized with an ABI PRISM 310 automated sequencer. Nucleotide sequences of newly described haplotypes were deposited into GenBank (Table I).

Sequences of the 5'-end (653 bp) and 3'-end (184 bp) of the CR (altogether 837 bp), 277 bp of the cytochrome *b* gene, 315 bp of the ATPase6 gene, *c.* 385 bp of *LDH-CI** and 572 bp of the ITS1 region were aligned using ClustalX (Thompson *et al.*, 1994) and compared with data previously published on Jadro softmouth and Neretva softmouth and brown trout.

A sequence evolution model was chosen using the programme MODELTEST 3.7 (Posada & Crandall, 1998) and used with PAUP 4.0b10 (Swofford, 2000). Phylogenetic analysis of mtDNA haplotypes characteristic of softmouth trout, along with those representing the main brown trout phylogenetic lineages, was performed using neighbour-joining (NJ) analysis based on selected distance in PAUP. Support for the nodes was obtained with 10 000 bootstrap replicates.

MICROSATELLITES

Twelve microsatellite loci, isolated and characterized from other salmonid species and optimized to amplify in two multiplex PCRs (8- and 4-plex), were chosen for analysis; for details of the loci, protocol and original references see Lerceteau-Köhler & Weiss

(2006). Aliquots of fluorescently labelled amplified DNA were mixed with formamide and GENESCAN-500 ROX Size Standard (AB Applied Biosystems) and genotyped on the ABI-310 with GeneScan™ Analysis Software 3.7.

Microsatellite allele frequencies, number of alleles per locus (A), observed (H_o) and expected (H_e) heterozygosities and exact probability tests for deviations from Hardy–Weinberg expectations (HWE) were performed with GENETIX 4.04 (Belkhir *et al.*, 1996–2004). All tests were conducted using 1000 permutations. FSTAT 2.9.3.2 (Goudet, 2001) was used to calculate allelic richness and pair-wise F_{ST} values. Corrections for multiple significance tests were made with a sequential Bonferroni-type correction (Rice, 1989).

Genetic relationships between individuals were estimated as the proportion of shared alleles at each locus, *i.e.* allele sharing distances (D_{AS}) (Bowcock *et al.*, 1994). A matrix of D_{AS} was used to construct a tree of individuals using NJ with POPULATIONS (Langella, 2002). Statistical support for major nodes in the tree was obtained with 1000 bootstrap replicates across loci. Global D_{AS} were obtained among populations and taxa.

The heterozygosity excess method, implemented in BOTTLENECK (Piry *et al.*, 1999) with the infinite allele, stepwise mutation and two-phase models (95% single-step mutations and 5% multiple-step mutations) were used to test for bottlenecks in population size in Vrljika and Neretva softmouth trout. The distribution of allele frequency classes was also examined for deviation from the L-shaped distribution for neutral loci at drift equilibrium. All analyses were performed with 10 000 iterations assuming mutation-drift equilibrium, and significances were calculated using the Wilcoxon signed-rank test.

RESULTS

SEQUENCE ANALYSIS

No sequence variation was found in Vrljika softmouth trout for either mtDNA or nuclear DNA, although this population was variable at some microsatellite loci (see below). Sequence alignment of combined mtDNA haplotypes of softmouth trout from the Vrljika, Neretva (haplotypes Soxy 1 and 2) and Jadro rivers indicated a close relationship between the haplotypes from the first two of these rivers, while the Jadro haplotype was most similar to brown trout haplotypes, as expected (see Introduction) (Fig. 2). Softmouth trout from Vrljika and Neretva differed by only one transition in the mtDNA CR and by two transitions in the ATPase6 gene (for accession numbers see Table I). No differences appeared between these populations in the cytochrome *b* gene, which was the least variable among the three mtDNA regions. The pair-wise HKY+I+G distance (selected by MODELTEST and inferred from combined mtDNA haplotypes) between Vrljika and Neretva softmouth trout was 0.27%, between Vrljika and Jadro softmouth trout as large as 2.76% and between Neretva and Jadro 2.42%.

Only slight differences among Vrljika, Neretva and Jadro softmouth trout were found among genomic DNA sequences, and these were restricted to the variable repeat region (cytosine-heavy stretch) of the *LDH-C1* IV* intron. Complete identity was observed in the rest of this intron and in the ITS1 region. No heterozygous individuals were found in softmouth trout.

MICROSATELLITES

Only 11 of 12 microsatellite loci were included in the statistical analysis: the allelic pattern of Ssosl417 could not be unambiguously determined in the three

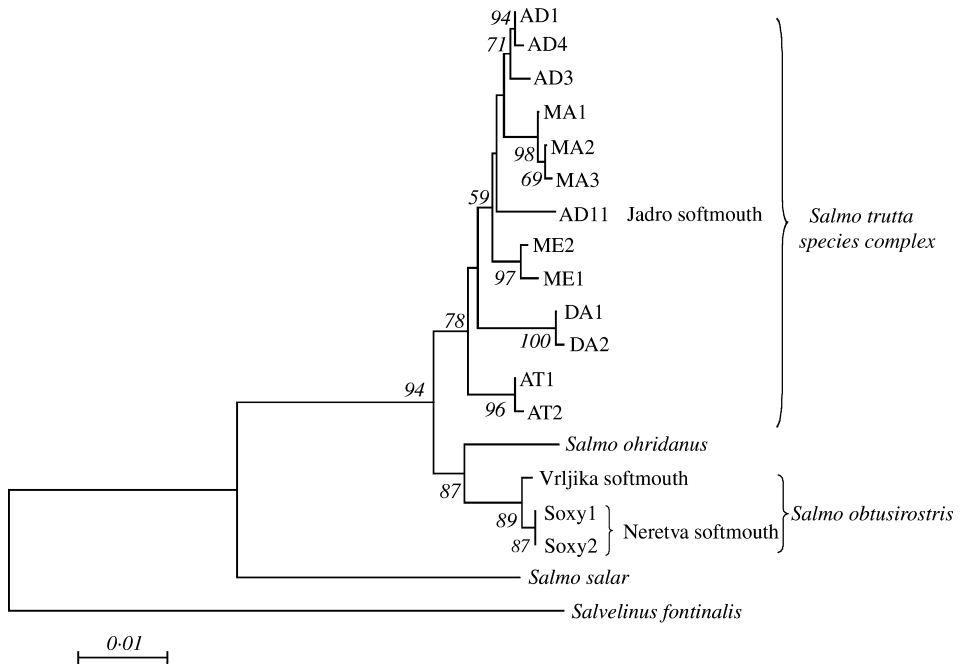


FIG. 2. Neighbour-joining tree for genus *Salmo*, including *Salmo obtusirostris* from the Jadro, Vrlika and Neretva (Soxy) rivers, *S. ohridanus* and major *Salmo trutta* phylogenetic lineages (AD, Adriatic; MA, *S. marmoratus*; ME, Mediterranean; DA, Danubian; AT, Atlantic) based on partial sequences of the mtDNA ATPase6 gene, cytochrome *b* gene and control region and the HKY+I+G substitution model (transition–transversion ratio: 5.3759; proportion of invariable sites = 0.6179; Gamma distribution shape parameter = 0.6251). The tree was rooted with *Salmo salar* and *Salvelinus fontinalis*. GenBank accession numbers available at www.balkan-trout.com/data.htm.

softmouth trout samples and was therefore removed from further analysis. Altogether, an average of 10.63 alleles per locus was found with Ssa85 monomorphic in all three softmouth trout populations.

For the other loci, the Neretva sample was the most polymorphic, exhibiting 102 alleles across 11 loci, while Jadro softmouth trout had 25 alleles and the Vrlika had 22 alleles (Table II). The three softmouth trout populations were in HWE, both within and across loci. Vrlika trout shared 16 alleles with the Neretva softmouth sample (Fig. 3) but only two with the Jadro trout sample. Alleles were fixed at six loci in Vrlika softmouth trout sample, while in Neretva softmouth trout, an average of seven alleles appeared across the six loci. Six private alleles distributed across three microsatellite loci appeared in Vrlika softmouth trout. The relationship between Vrlika and Neretva softmouth trout was reflected in small D_{AS} and F_{ST} values (Table III) and was closer than that between Vrlika and Jadro softmouth trout.

Genetic distances between individuals, including Neretva brown trout (Fig. 4), revealed three distinctive clusters corresponding to Neretva softmouth, Jadro softmouth and Neretva brown trout. Individuals of Vrlika softmouth trout were clearly separated from other individuals within the Neretva softmouth trout cluster.

TABLE II. Sample size (n), number of alleles (A), allelic richness (AR), observed (H_o) and expected (H_e) heterozygosity and F_{IS} values. For Neretva brown trout, locus Ssa410 was treated as missing data due to no or insufficient amplification

Locus	n	Softmouth trout			Brown trout
		Vrljika 23	Neretva 33	Jadro 10	Neretva 21
Str60	A	1	2	1	2
	AR	1.000	1.242	1.000	2.000
	H_o	NA	0.030	NA	0.571
	H_e	NA	0.030	NA	0.444
	F_{IS}	NA	0	NA	-0.263
Ssos1438	A	1	7	3	4
	AR	1.000	4.245	2.968	3.372
	H_o	NA	0.546	0.500	0.524
	H_e	NA	0.571	0.540	0.455
	F_{IS}	NA	0.060	0.126	-0.128
Ssa85	A	1	1	1	3
	AR	1.000	1.000	1.000	2.337
	H_o	NA	NA	NA	0.238
	H_e	NA	NA	NA	0.285
	F_{IS}	NA	NA	NA	0.187
SSsp2216	A	2	7	3	10
	AR	2.000	5.554	3.000	6.971
	H_o	0.391	0.909	0.500	0.762
	H_e	0.466	0.792	0.656	0.812
	F_{IS}	0.182	-0.133	0.300	0.086
Str73	A	1	3	1	4
	AR	1.000	1.485	1.000	3.782
	H_o	NA	0.061	NA	0.238
	H_e	NA	0.059	NA	0.604
	F_{IS}	NA	-0.008	NA	0.621***
Ssa410	A	2	21	4	NA
	AR	1.937	10.924	3.993	NA
	H_o	0.261	0.849	0.778	NA
	H_e	0.227	0.931	0.661	NA
	F_{IS}	-0.128	0.104	-0.120	NA
Ssa408	A	8	17	5	15
	AR	6.007	9.289	4.869	9.009
	H_o	0.870	0.939	0.556	0.810
	H_e	0.775	0.895	0.586	0.874
	F_{IS}	-0.100	-0.034	0.111	0.098
Ssa-D190	A	1	6	2	12
	AR	1.000	4.989	2.000	7.017
	H_o	NA	0.742	0.400	0.750
	H_e	NA	0.771	0.320	0.698
	F_{IS}	NA	0.053	-0.200	-0.050

TABLE II. Continued

Locus	<i>n</i>	Softmouth trout			Brown trout
		Vrļjika 23	Neretva 33	Jadro 10	Neretva 21
SSsp2213	<i>A</i>	1	10	2	12
	<i>AR</i>	1.000	6.185	1.800	7.879
	<i>H_o</i>	NA	0.677	0.100	0.905
	<i>H_c</i>	NA	0.712	0.095	0.832
	<i>F_{IS}</i>	NA	0.065	0	-0.063
Ssa-D71	<i>A</i>	1	14	1	14
	<i>AR</i>	1.000	8.805	1.000	9.461
	<i>H_o</i>	NA	0.879	NA	1.000
	<i>H_c</i>	NA	0.877	NA	0.894
	<i>F_{IS}</i>	NA	0.014	NA	-0.094
OMM106	<i>A</i>	3	14	2	12
	<i>AR</i>	2.358	9.158	2.000	7.438
	<i>H_o</i>	0.318	0.903	0.300	0.263
	<i>H_c</i>	0.385	0.902	0.455	0.803
	<i>F_{IS}</i>	0.197	0.015	0.386	0.687
Overall	<i>A</i>	2.000	9.273	2.273	8.800
	<i>AR</i>	1.755	5.716	2.239	5.927
	<i>H_o</i>	0.167	0.594	0.285	0.606
	<i>H_c</i>	0.169	0.594	0.301	0.670
	<i>F_{IS}</i>	0.030	0.016	0.112	0.120**

Significances after Bonferroni-type corrections: **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Tests for recent bottlenecks failed to show significant heterozygosity excesses in Vrļjika and Neretva softmouth trout population. The mode-shift indicator was consistent with an L-shaped allele frequency distribution in both samples.

DISCUSSION

MOLECULAR PHYLOGENY AND DIVERSITY OF VRLJKA SOFTMOUTH TROUT

Evolutionary inferences made on the basis of mtDNA alone suggest a sister relationship between Vrļjika and Neretva softmouth trout and much more distant relationships between these species and Jadro softmouth trout. However, this conclusion may be misleading because the Jadro softmouth trout mitochondrial genome appears to be of brown trout origin due to an ancient hybridization (Sušnik *et al.*, 2007b). The only inference that can be made from the mtDNA data is that present-day populations of Vrļjika softmouth trout probably did not evolve from Jadro softmouth after the latter had been introgressed by brown trout mtDNA. The use of mtDNA variation to investigate phylogenetic relationships among softmouth trout from the rivers Jadro, Vrļjika and Neretva, therefore, may be inappropriate as the timing of the hybridization cannot be unambiguously determined.

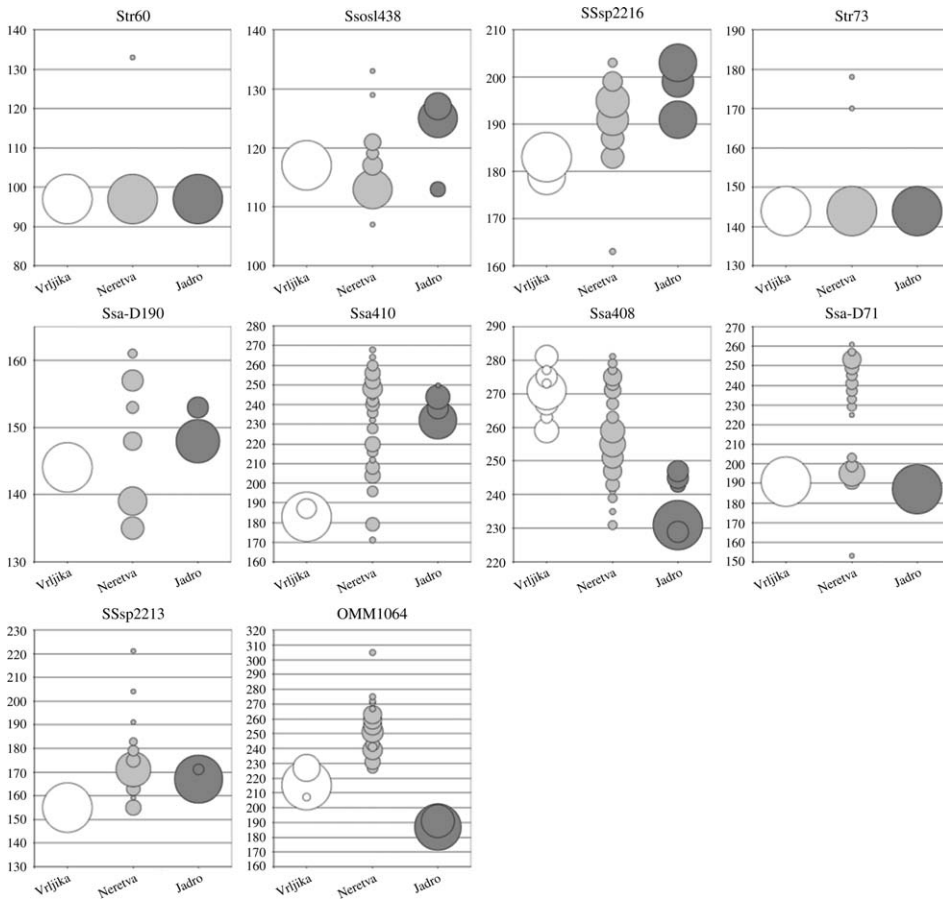


FIG. 3. Graphical representation of allele size in base pairs (y-axis) and frequency distributions (size of bubble corresponds to allele frequency) at 10 microsatellite loci in Vrljika, Neretva and Jadro softmouth trout. Microsatellite locus Ssa85 was monomorphic in all samples and is not included.

More reliable information, indicating a closer relationship between Vrljika and Neretva softmouth trout than between softmouth trout in the Vrljika and Jadro rivers, appears in the microsatellite DNA analysis, which detected considerable allele sharing between Vrljika and Neretva softmouth trout along with a tree topology that supported their monophyletic origin (Fig. 4). Given the geomorphologic characteristics of the region (see Introduction) inhabited by the three softmouth trout populations, Vrljika softmouth trout probably do not stem from the Jadro population but rather from populations in the River Neretva. If so, the Jadro and Vrljika populations cannot be considered the same taxon, *i.e.* *S. o. salonitana*, as proposed by Mrakovčić *et al.* (2006).

Despite the evidently close relationship between Vrljika and Neretva softmouth trout, some mtDNA and microsatellite DNA differences indicate that these two populations did not separate recently.

The Vrljika haplotype appears to have evolved as a consequence of a mutation event giving rise to a derived character state or represents a reminder of

TABLE III. Matrix of pair-wise F_{ST} values (above diagonal) and their significance and allele sharing distances (D_{AS}) based on microsatellite data

	Vrljika softmouth trout	Neretva softmouth trout	Jadro softmouth trout	Neretva brown trout
Vrljika softmouth trout		0.407**	0.692**	0.553**
Neretva softmouth trout	0.491		0.290**	0.323**
Jadro softmouth trout	0.682	0.491		0.436**
Neretva brown trout	0.888	0.764	0.863	

** $P < 0.01$, after correction for multiple tests.

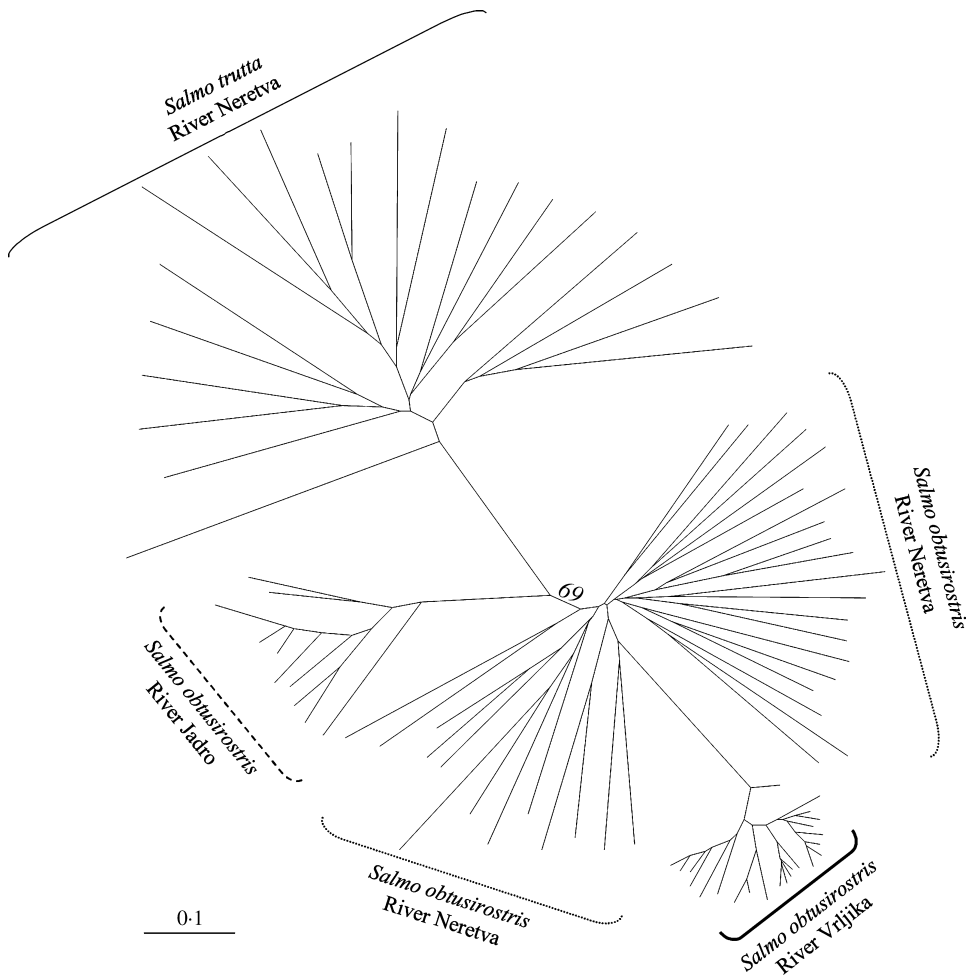


FIG. 4. Neighbour-joining tree of individuals based on D_{AS} estimated from eleven microsatellite loci.

ancestral polymorphism that has been due to genetic drift and bottleneck fixed in the Vrljika and lost in the Neretva softmouth trout. In either case, the DNA sequence of the Vrljika haplotype provides strong evidence that this population originated naturally rather than as a consequence of anthropogenic activities (e.g. translocations), which have been instrumental in determining phylogeographic patterns and the genetic diversity in other trout populations (Jug *et al.*, 2005; Sanz *et al.*, 2006; Razpet *et al.*, 2007).

Assuming that the mtDNA haplotype in Vrljika softmouth trout evolved from Neretva populations and assuming a salmonid molecular clock of 1–2% per million years (Bernatchez, 2001), these populations appear to have diverged from each other in the late Pleistocene (135 000–270 000 years ago). This time scale is comparable to estimated ages of various phylogeographic lineages of brown trout, including *Salmo platycephalus* Behnke and *Salmo (trutta) marmoratus* Cuvier, which are regarded by some authors as distinct species (Kottelat, 1997; Sušnik *et al.*, 2004).

Low average number of alleles per microsatellite locus, high number of fixed loci and very low heterozygosity (Table II) in Vrljika softmouth trout suggest a reduction in genetic diversity that often appears in small fragmented populations. A reduction in genetic diversity following population subdivision has the potential to affect population survival significantly (Keller & Waller, 2002; Spielman *et al.*, 2004; Frankham, 2005). Moreover, environmental factors may be of greater importance than genetic factors for the survival of fragmented populations (Caro & Laurenson, 1994). Evidence for this view has recently emerged from studies of geographically separated small populations of marble *Salmo marmoratus* and brown trout in Slovenia (Fumagalli *et al.*, 2002; Snoj, 2004) and of a translocated softmouth trout population in the River Žrnovnica, Croatia (Snoj *et al.*, 2007). By analogy, the same may hold true for the Vrljika population, which appears to have maintained its viability over a period of several tens of thousands of generations despite low genetic variability.

EVOLUTION OF THE VRLJIKA SOFTMOUTH TROUT

An obvious explanation for the present geographic distribution and genetic diversity of softmouth trout in the Rivers Vrljika and Neretva is that the former was colonized by individuals from the latter at some time in the past *via* direct communication through the River Trebižat (Fig. 1). The present genetic structure of Vrljika and Neretva softmouth trout is probably a consequence of vicariance, which split a formerly continuous water system populated by a common ancestor into large (Neretva) and small (Vrljika) subdivisions. The absence of shared mtDNA haplotypes indicates that communication between these populations has not been recently re-established. Given that these two rivers are now connected by only subterranean flows, questions arise as to what kind of mechanism enabled such a communication, how long it lasted and what finally blocked it. The most parsimonious explanation is that the Vrljika and Trebižat rivers once shared a continuous surface water system enabling the migration of fish upstream and downstream until the lower part of the present-day River Vrljika went underground through karstification or tectonic shift or both. However, the geological evidence contradicts this hypothesis,

indicating that the physical barrier separating the sink-holes of the River Vrljika at the eastern part of Imotsko Polje and the River Trebižat are several million years old (Slišković & Ivičić, 1999). This date considerably precedes the Pleistocene Epoch, when most *Salmo* taxa first appeared and when fragmentation of softmouth species likely occurred. Thus, subterranean dispersal would appear to be the most probable explanation for the appearance of softmouth trout in the River Vrljika.

To the authors knowledge, there is no literature available on subterranean migration of above-ground fish. In the case of Vrljika softmouth trout, the question arises as to how the fish moved from a lower to an upper level. Did they actively migrate upstream underground or were they assisted by other mechanisms facilitating movement? Temporary reversals of subterranean water currents, for example, can occur in karstic terrains. Underground water movements are complex, and natural U-shaped siphons can cause water to flow upstream and emerge as springs called 'estavelle' (Jović, 2003). Water flow from estavelles depends on the transient nature of subterranean passages, which is strongly associated with water level in the stream or river. During the Pleistocene, the alternation of glacial and interglacial phases caused great fluctuations in water level and might have, along with tectonic collapse of subterranean walls or deposition of calcareous layers, or both, subsequently blocked previous communication channels and subterranean migration of fish between the Rivers Vrljika and Neretva.

In conclusion, this study provides the first genetic data on Vrljika softmouth trout to reconstruct a phylogeny and to assess the taxonomy of this species. These results are of particular importance, given that this population has been regarded as a geographic variant of the Jadro softmouth trout and classified as *Salmo obtusirostris salonitana* (Mrakovčić *et al.*, 2006).

Morphological data are not yet available to compare Vrljika and Neretva softmouth trout in detail, but their overall external appearances clearly indicate that they represent phenotypically different forms. Although these phenotypic differences may be environmentally influenced, it is clear that Vrljika softmouth trout have remained reproductively isolated from the Neretva populations for several tens of thousands of years. During this time, the former fish have undergone sufficient genetic differentiation to be distinguished at the molecular level by a unique combination of character states.

The Vrljika softmouth trout is the only known softmouth trout population that does not coexist with brown trout; all other softmouth trout populations, including those in the Neretva (Snoj *et al.*, 2002), Jadro (Sušnik *et al.*, 2007b) and Zeta (Sušnik *et al.*, 2007a) rivers, appear to live, temporarily or permanently, in sympatry with brown trout. As a result, some introgressive hybridization has occurred between brown trout and softmouth trout in these latter populations (Razpet *et al.*, 2007). Vrljika trout may represent the last collection of genetically unmodified softmouth trout genomes, but because of its restricted habitat and small population size, its survival is under constant threat. Given its genetic uniqueness, this population deserves to be protected and given special consideration for conservation.

Vrljika softmouth trout should be formally recognized as a separate taxon, so that appropriate management and conservation actions can be taken. Available

data on softmouth trout depict a group of taxa composed of a limited number of phenotypically distinct populations that can also be differentiated at the molecular level, except perhaps the unstudied Krka populations. Future comparative studies, including both genetic and morphological analyses from all five softmouth trout populations, will likely show that *S. obtusirostris* includes more than one species. The results of the present study indicate that Vrljika softmouth trout should be given the same taxonomic status as the other geographically separated populations and recognized as a fifth entity within the species complex.

Authors thank I. Wilson, B. Delling, A. Mikulić, Fisheries Institute of Slovenia and P. Marijanović. This work was financed by Patagonia World Trout project (<http://www.flyfishusa.com/apparel/patagonia/patagonia-world-trout.htm>).

References

- Avise, J. C. (2000). *Phylogeography*. Cambridge: Harvard University Press.
- Bernatchez, L. (2001). The evolutionary history of brown trout (*Salmo trutta* L.) inferred from phylogeographic, nested clade, and mismatch analyses of mitochondrial DNA variation. *Evolution* **55**, 351–379.
- Bernatchez, L. & Danzmann, R. G. (1993). Congruence in control-region sequence and restriction-site variation in mitochondrial DNA of brook charr (*Salvelinus fontinalis* Mitchell). *Molecular Biology and Evolution* **10**, 1002–1014.
- Bogutskaya, N. G. & Zupančić, P. (2003). *Phoxinellus pseudalepidotus* a new species from the Neretva basin with an overview of the morphology of *Phoxinellus* species of Croatia and Bosnia-Herzegovina. *Ichthyological Exploration of Freshwaters* **14**, 369–383.
- Bowcock, A. M., Ruiz-Linares, A., Tomfohrde, J., Minch, E., Kidd, J. R. & Cavalli-Sforza, L. L. (1994). High resolution of human evolutionary trees with polymorphic microsatellites. *Nature* **31**, 455–457.
- Branco, M., Monnerot, M., Ferrand, N. & Tempelton, A. R. (2002). Postglacial dispersal of the European rabbit (*Oryctolagus cuniculus*) on the Iberian Peninsula reconstructed from nested glade and mismatch analyses of mitochondrial DNA genetic variation. *Evolution* **56**, 792–803.
- Caro, T. M. & Laurenson, M. K. (1994). Ecological and genetic factors in conservation – a cautionary tale. *Science* **263**, 485–486.
- Cronin, M. A., Spearman, W. J. & Wilmot, R. L. (1993). Mitochondrial variation in Chinook (*Oncorhynchus tshawytscha*) and chum salmon (*O. keta*) detected by restriction enzyme analysis of polymerase chain reaction (PCR) products. *Canadian Journal of Fisheries and Aquatic Sciences* **50**, 708–715.
- Frankham, R. (2005). Genetics and extinction. *Biological Conservation* **126**, 131–140.
- Freyhof, J., Lieckfeldt, D., Bogutskaya, G. N., Pitra, K. & Ludwig, A. (2006). Phylogenetic position of the Dalmatian genus *Phoxinellus* and description of the newly proposed genus *Delminichthys*. *Molecular Phylogenetics and Evolution* **38**, 416–425.
- Fumagalli, L., Snoj, A., Jesenšek, D., Balloux, F., Jug, T., Duron, O., Brossier, F., Crivelli, A. J. & Berrebi, P. (2002). Extreme genetic differentiation among the remnant populations of marble trout (*Salmo marmoratus*) in Slovenia. *Molecular Ecology* **11**, 2711–2716.
- Giuffra, E., Bernatchez, L. & Guyomard, R. (1994). Mitochondrial CR and protein coding genes sequence variation among phenotypic forms of brown trout *Salmo trutta* from northern Italy. *Molecular Ecology* **3**, 161–171.
- Jović, V. (2003). Vode zabiokovskog dijela neretvanskog sliva: istražnost i prijedlog daljnjih aktivnosti. Symposium “Voda u kršu Cetine, Neretve i Trebišnice”, Neum, *Zbornik radova* **25–27**, 18–30.

- Jug, T., Berrebi, P. & Snoj, A. (2005). Distribution of non-native trout in Slovenia and their introgression with native trout populations as observed through micro-satellite DNA analysis. *Biological Conservation* **123**, 381–388.
- Keller, L. F. & Waller, D. M. (2002). Inbreeding effects in wild populations. *Trends in Ecology and Evolution* **17**, 230–241.
- Kottelat, M. (1997). European freshwater fishes. *Biologia* **52** (Suppl. 5), 1–271.
- Lerceteau-Köhler, E. & Weiss, S. (2006). Development of multiplex PCR microsatellite assay in brown trout *Salmo trutta*, and its potential application for the genus. *Aquaculture* **258**, 641–645.
- Mayr, E. (1942). *Systematics and the Origin of Species*. New York, NY: Columbia University Press.
- Mayr, E. (1963). *Animal Species and Evolution*. Cambridge: Harvard University Press.
- McMeel, O. M., Hoey, E. M. & Ferguson, A. (2001). Partial nucleotide sequences, and routine typing by polymerase chain reaction-restriction fragment length polymorphism, of the brown trout (*Salmo trutta*) lactate dehydrogenase, *LDH-C1*90* and **100* alleles. *Molecular Ecology* **10**, 29–34.
- Miller, S. A., Dykes, D. D. & Polesky, H. F. (1988). A simple salting out procedure from human nucleated cells. *Nucleic Acids Research* **16**, 1215.
- Mrakovčić, M. & Mišetić, S. (1990). Status, distribution and conservation of the salmonid, *Salmothymus obtusirostris* (Heckel) and cyprinid, *Aulopyge hugely* (Heckel) in Yugoslavia. *Journal of Fish Biology* **37** (Suppl.), 241–242.
- Mrakovčić, M., Brigić, A., Buj, I., Caleta, M., Mustafić, P. & Zanella, D. (2006). *Red Book of Freshwater Fish in Croatia*. Zagreb: Ministry of Culture, State Institute for Nature Protection.
- Page, R. D. M. & Holmes, E. C. (2001). *Molecular Evolution*. Oxford: Blackwell Science.
- Phillips, R. P., Matsuoka, M. P., Konon, I. & Reed, K. M. (2000). Phylogenetic analysis of mitochondrial and nuclear sequences supports inclusion of *Acantholingua ohridana* in the genus *Salmo*. *Copeia* **2000**, 546–550.
- Piry, S., Luikart, G. & Cornuet, J. M. (1999). BOTTLENECK: a computer program for detecting recent reductions in the effective population size using allele frequency data. *Journal of Heredity* **90**, 502–503.
- Posada, D. & Crandall, K. A. (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**, 817–818.
- Presá, P., Pardo, B. G., Martínez, P. & Bernatchez, L. (2002). Phylogeographic congruence between mtDNA and rDNA ITS markers in brown trout. *Molecular Biology and Evolution* **19**, 2161–2175.
- Razpet, A., Sušnik, S., Jug, T. & Snoj, A. (2007). Genetic variation among trout in the River Neretva basin, Bosnia and Herzegovina. *Journal of Fish Biology* **70**, 94–110.
- Rice, W. R. (1989). Analysing tables of statistical tests. *Evolution* **43**, 223–225.
- Sanz, N., Cortey, M., Pla, C. & Garzia-Marin, J. L. (2006). Hatchery introgression blurs ancient hybridisation between brown trout (*Salmo trutta*) lineages as indirect by complementary allozymes and mtDNA markers. *Biological Conservation* **130**, 278–289.
- Schöffmann, J. (2003). Zur aktuellen Situation der vier Unterarten der Weichmaulforelle, *Salmo (Salmothymus) obtusirostris* Heckel 1851. *Österreich Fischerei* **56**, 180–184.
- Schöffmann, J. (2004). Nachweis einer Restpopulation der bereits für ausgestorben erklärten Weichmaulforelle, *Salmo (Salmothymus) obtusirostris* ssp., der Vrljika, südliches Kroatien. *Österreich Fischerei* **57**, 277–278.
- Seehausen, O. (2004). Hybridization and adaptive radiation. *Trends in Ecology and Evolution* **19**, 198–207.
- Slišković, I. & Ivičić, D. (1999). Trebižat river catchment hydrogeology and water use. In *2nd Croatian Conference on Waters* (Gereš, D., ed.), pp. 589–595. Zagreb, Croatia: Hrvatske Vode.
- Snoj, A. (2004). Filogeografska struktura postrvi (*Salmo trutta* L.) v Sloveniji. *Ribič* **10**, 239–243.

- Snoj, A., Jug, T., Melkič, E., Sušnik, S., Jesenšek, D., Budihna, N., Pohar, J. & Dovč, P. (2000). Mitochondrial and microsatellite DNA analysis of marble trout in Slovenia. *Journal of Freshwater Biology (Quaderni ETP)* **29**, 5–11.
- Snoj, A., Melkič, E., Sušnik, S., Muhamedagić, S. & Dovč, P. (2002). DNA phylogeny supports revised classification of *Salmothymus obtusirostris*. *Biological Journal of the Linnean Society* **77**, 399–411.
- Snoj, A., Tomljanović, T., Razpet, A., Treer, T. & Sušnik, S. (2007). Genetic composition of the Jadro softmouth trout following translocation into a new habitat. *Conservation Genetics* **8**, 1213–1217. doi: 10.1007/s10592-006-9262-2.
- Spielman, D., Brook, B. W. & Frankham, R. (2004). Most species are not driven to extinction before genetic factors impact them. *Proceeding of the National Academy of Science of the United States of America* **101**, 15261–15264.
- Sušnik, S., Schöffmann, J. & Snoj, A. (2004). Phylogenetic position of *Salmo (Platysalmo) platycephalus* Behnke 1968 from south-central Turkey, evidenced by genetic data. *Journal of Fish Biology* **64**, 947–960.
- Sušnik, S., Snoj, A., Wilson, I., Mrdak, D. & Weiss, S. (2007a). Historical demography of brown trout (*Salmo trutta*) in the Adriatic drainage including the putative *S. letnica* endemic to lake Ohrid. *Molecular Phylogenetics and Evolution* **44**, 63–76.
- Sušnik, S., Weiss, S., Odak, T., Delling, B., Treer, T. & Snoj, A. (2007b). Reticulate evolution: ancient introgression of the Adriatic brown trout mtDNA in softmouth trout *Salmo obtusirostris* (Teleostei: Salmonidae). *Biological Journal of the Linnean Society* **90**, 139–152.
- Swofford, D. L. (2000). *PAUP*, b-VERSION 4.0*. Sunderland, MA: Sinauer.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4637–4680.
- Verovnik, R., Sket, B. & Trontelj, P. (2004). Phylogeography of subterranean and surface populations of water lice *Asellus aquaticus* (Crustacea: Isopoda). *Molecular Ecology* **13**, 1519–1532.

Electronic References

- Belkhir, K., Borsa, P., Chikhi, L., Raufaste, N. & Bonhomme, F. (1996–2004). *GENETIX 4.05, logiciel sous Windows TM pour la genétique des populations*. Montpellier: Laboratoire Genome, Populations, Interactions, CNRS UMR 5000, Université de Montpellier II. Available at <http://www.univ-montp2.fr/~genetix>
- Goudet, J. (2001). *FSTAT, a program to estimate and test gene diversities and fixation indices (Version 2.9.3)*. Available at <http://www.unil.ch/izea/software/fstat.html>
- Langella, O. (2002). *Populations, 1.2.30. Copyright (C) 1999, Olivier Langella, CNRS UPR9034*. Available at <http://bioinformatics.org/~tryphon/populations/>