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Genetic and morphological characterization of a Lake Ohrid endemic, *Salmo (Acantholingua) ohridanus* with a comparison to sympatric *Salmo trutta*

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Analysis of both uni- (two mtDNA gene sequences) and bi-parentally (seven microsatellite loci) inherited genetic markers, together with analysis of 40 morphological characters, described *Salmo ohridanus* as a highly divergent member of the genus *Salmo*. Based on comparative substitution rate differences at the cytochrome *b* gene, and a rough estimated age of the *Salmo trutta* complex (*i.e.* at least 2 million years), the *S. ohridanus* and *Salmo obtusirostris* clade probably split from a common ancestor of brown trout *Salmo trutta* >4 million years ago, overlapping with minimum age estimates of the formation of Europe's oldest freshwater habitat, Lake Ohrid. Comparative analysis with Lake Ohrid brown trout (known regionally as *Salmo letnica*), supported the notion that these fish have more recently colonized the lake and phylogenetically belong to the Adriatic lineage of brown trout. It is further suggested that species-specific saturation in the mtDNA control region underestimated the divergence between *S. ohridanus* and *S. trutta*. Evidence of rare hybridization between *S. ohridanus* and Lake Ohrid brown trout was seen at both mtDNA and microsatellite markers, but there was no support for extensive introgression.

Key words: Adriatic drainage; belvica; cytochrome *b* gene; microsatellies; mtDNA control region; *Salmo trutta*.

INTRODUCTION

The genus *Salmo* contains two of Europe's most economically important and well-researched fishes, brown trout *Salmo trutta* L. and Atlantic salmon *Salmo salar* L. Numerous additional taxa in the genus have been recognized (Kottelat, 1997), but nearly all belong to what some consider the *S. trutta* species complex (Bernatchez *et al.*, 1992; Bernatchez, 2001). Recently, two highly distinct salmonid taxa, clearly divergent from this complex, were reclassified as species within the genus *Salmo*: *Salmo obtusirostris* (Heckel) and *Salmo ohridanus* Steindachner (Phillips, *et al.*, 2000; Snoj *et al.*, 2002). Both species are endemic to the Adriatic

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drainage system, which has received little attention in terms of salmonid phylogenetic studies. The Balkan region is considered a global 'hotspot' of biodiversity (Conservation International, http://www.conservation.org/xp/CIWEB/home). The Adriatic drainage in the Balkans served as a refuge for freshwater fishes during the Messinian crisis (Bianco, 1990) and throughout the Pleistocene when periodic glaciations affected most other European river systems. Moreover, the region contains one of the most unique yet understudied bodies of fresh water in Europe, Lake Ohrid, Republic of Macedonia. Lake Ohrid is Europe's oldest lake and is believed to have been formed as early as during the Tertiary period (4–10 million years ago; Bănărescu, 1991) as a result of tectonic shifts. Due to its southern geographic location, it was not directly affected by Pleistocene glaciations and hence is characterized by unique flora and fauna distinct to a pre-glaciation period (Stanković, 1960; Bănărescu, 2004; Frogley & Preece, 2004; Korniushin, 2004). Ancient primary lake fauna and more recent elements of the Adriatic or even Danubian freshwater fauna meet in Lake Ohrid (Stanković, 1960; Šorić, 1990; Bănărescu, 1991; Martens *et al*., 1994; Griffiths & Frogley, 2004). Among salmonids, Lake Ohrid brown trout *Salmo letnica* (Karaman) represents an element of the Adriatic freshwater fauna as it was clearly placed into the Adriatic cluster of a *S. trutta* mtDNA phylogeny (*S. trutta*, Bernatchez, 2001 and Apostolidis, *et al*. 1997; *S. letnica*, Sell & Spirkovski, 2004). The study of Sell & Spirkovski (2004) also presents some support for the existence of two reproductively isolated units within this taxon. In contrast to this presumably modern lineage, *S. ohridanus* represents a lineage highly divergent from *S. trutta*. *Salmo ohridanus*, known locally as belvica (white fish), has had a varied taxonomic history. It was placed in the same genus (*Salmothymus*) (Berg, 1910) as the Adriatic endemic softmouth trout (*S. obtusirostris*), later placed in a monotypic genus as *Acantholingua ohridana* (Hadžišče, 1961), recently reclassified by Phillips *et al*. (2000) as *Salmo ohridana* (properly *S. ohridanus*, thus completing a 'cycle' as it assumes the original name given by Steindachner), and now viewed as a sister species to softmouth trout *Salmo obtusirostris* (Snoj *et al*., 2002).

Salmo ohridanus inhabits deep parts of the lake (40–60 m). It is characterized by a relatively elongated body form with a silver colour, little or no spotting, and a deeply forked tail. Apart from the phylogenetic analyses of the same two individuals in Phillips *et al*. (2000, 2004) as well as Snoj *et al*. (2002) and the morphological notes of Hadžišče (1960, 1961) no characterization of this highly unique *Salmo* species exists in the modern literature. The aim of the present study was to characterize *S. ohridanus* and its potential relation to the congeneric Lake Ohrid brown trout, using both bi-parentally and uni-parentally inherited molecular markers as well as meristic and morphometric measurements.

MATERIAL AND METHODS

SAMPLES AND DNA EXTRACTION

In 2004, 54 fish (30 *S. ohridanus* and 24 Lake Ohrid brown trout) were purchased through commercial fisherman on the Macedonian shores of Lake Ohrid. According to fisherman, the Lake Ohrid brown trout samples represented the winter form (known

locally as *S. letnica typicus*). Total DNA was isolated from fin clips, preserved in ethanol, using a high-salt extraction technique (Miller *et al.*, 1988).

MTDNA ANALYSIS

The complete mtDNA control region (CR) was amplified in 30 *S. ohridanus* and 24 Lake Ohrid brown trout using primers 28RIBa (Snoj *et al.*, 2000) and HN20 (Bernatchez & Danzmann, 1993). A part of the cytochrome *b* gene (cyt b) was amplified in 11 *S. ohridanus* and four Lake Ohrid brown trout using the primers H15149 and L14841 (Kocher *et al.*, 1989). Polymerase chain reactions (PCR) were carried out in 25 μL volumes. Each reaction contained 0·5 μM of each primer, 0·2 mM dNTP, 1·5 mM $MgCl_2$, 1X PCR buffer, 1 U of *Taq* polymerase (PE Applied Biosystems Fostercity, CA, U.S.A.) and 50–100 ng of genomic DNA. The PCR conditions were as follows: initial denaturation at 94° C for 3 min, followed by denaturation at 94° C (45 s), annealing at 53° C (for CR) or 52° C (for cyt b) (30 s) and extension at 72° C (45 s) repeated for 30 cycles.

Amplified DNA fragments were run on a 1·5% agarose gel, were cut out of the gel, and purified using the QIAEX II Gel Extraction Kit (QIAGEN, PE Applied Biosystems). Approximately 100 ng of purified PCR product was used in cycle sequencing reactions following ABI PRISM BigDye Terminator protocols (PE Applied Biosystems). Sequences were visualized on an ABI-3100 automated capillary sequencer and aligned using the computer programme ClustalX (Thompson *et al.*, 1994). New mtDNA sequences generated in this study have been deposited in GenBank (Table I).

Aligned sequences were imported into the programme PAUP Version 4·0b10 (Swofford, 2000) for phylogenetic analysis. Neighbour-Joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) were used for phylogenetic reconstruction. For NJ, a Kimura 2-parameter model was chosen. For MP, insertions or deletions (indels) were included as a fifth character. A heuristic search (10 replicates) with tree-bisection-reconnection (TBR) branchswapping was employed to find the most parsimonious trees. For ML, a sequence evolution model was first chosen using the programme Modeltest Version 3·06 (Posada & Crandall, 1998) incorporated into PAUP. After a model was chosen, a heuristic search (10 replicates) was used to estimate the most likely topology. Support values for the nodes were obtained with 1000 bootstrap replicates for MP, NJ or ML analysis, whereby the fast stepwise addition method was used for ML. Pairwise divergence between haplotypes and net divergence among groups of haplotypes was calculated using a Kimura 2 parameter algorithm in MEGA2 (Kumar *et al.*, 2001).

MICROSATELLITES

Seven microsatellite loci, isolated and characterized from other salmonid species were chosen for analysis. The names of these loci, there GenBank accession numbers and literature references are given as follows: BFRO001, U90327 (Snoj *et al.*, 1997); Ssa197, U43694 (O'Reilly *et al.*, 1996); Strutta 58, U60223 (Poteaux *et al.*, 1999); Ssosl438, Z49134 (Slettan *et al.*, 1996); Ssa85; U43692 (O'Reilly *et al.*, 1996); OMM1064, AF352744 (Rexroad *et al.*, 2002); SsoSL417, Z48598 (Slettan *et al.*, 1995).

All forward primers were fluorescently labelled. Polymerase chain reactions were performed in 10 μl reaction mixtures containing 0·5 μM of each primer, 0·2 mM dNTP, 1·5 mM $MgCl_2$, 1x PCR buffer, 0·5 U of *Taq* polymerase (PE Applied Biosystems) and 50 ng of genomic DNA. PCR profile comprised initial DNA denaturation (95° C, 3 min), and 30 successive cycles of strand denaturation (94° C, 45 s), primer annealing (54° C, 15 s) and DNA extension (72° C, 5 s). Aliquots of fluorescently labelled amplified DNA were mixed with formamide and GENESCAN-350 ROX Size Standard (PE Applied Biosystems) and genotyped on the ABI-3100 using the GeneScan^{TM} Analysis Software 3·7.

Microsatellite allele frequencies, the number of alleles per locus (A), observed and expected heterozygosities and exact probability tests for deviations from

TABLE I. GeneBank accession numbers for mtDNA control region and cytochrome *b* gene haplotypes used for phylogenetic analysis

	GeneBank Accession number	
Taxon/haplotype	Control region	Cytochrome *b* gene
Salmo ohridanus		
Haplo1	AY926564	AF053590
Haplo2	AY926560	AF053590
Haplo3	AY926568	AF053590
Haplo4	AY926561	AF053590
Haplo5	AY926569	AF053590
Haplo6	AY926559	AF053590
Haplo7	AY926563	AF053590
Haplo8	AY926567	AF053590
Haplo9	AY926565	AF053590
Haplo10	AY926562	AF053590
Haplo11	AY926566	AF053590
Lake Ohrid brown trout		
Haplo12	AY926570	U63892
Haplo13	AY926573	U63892
Haplo14	AY926571	U63892
Haplo15	AY926572	U63892
Salmo trutta		
MAcs1	AY836365	X76251
MEcs1	AY836350	X76248
MEcs2	AY836351	X76249
ADcs1	AY836330	X76250
ADcs2	AY836331	X76251
ADcs3	AY836332	X76251
DA1a	AY185568	X76252
DA2	AY185570	X76252
AT1	AF273086	X76254
AT2	AF273087	X76254
Salmo obtusirostris	AF488535	AF488534
Salmo salar	AF133701	AF133701
Oncorhynchus mykiss	NC_001717	NC_001717

Hardy-Weinberg equilibrium (HWE) and deviations from genotypic linkage equilibrium (LE) were performed using the programme GENETIX 4·04 (Belkhir *et al*., 1996–2004). All tests were conducted using 10000 permutations. The frequency of potential null alleles was estimated using the approach of Brookfield (1996). Locus specific and overall R_{st} (following Rousset, 1996 and Goodman, 1997) and pairwise F_{st} values were calculated in FSTAT 2.9.3.2 (Goudet, 2001) and a sequential Bonferoni-type correction (Rice, 1989) was applied for multiple significance tests.

MORPHOLOGICAL ANALYSIS

Thirty-tree individuals of *S. ohridanus* and 16 of Lake Ohrid brown trout were included in morphological analyses. Morphological characterization was based on 12

meristic and 28 morphometric characters. The meristic characters were: (i) pores along the lateral line, (ii) unbranched dorsal rays, (iii) branched dorsal rays, (iv) total number of rays, (v) branched pectoral rays, (vi) ventral rays, (vii) unbranched anal rays, (viii) branched anal rays, (ix) gill rakers on the first left branchial arch, (x) branchiostegal rays, (xi) vertebrae (with central and haemal spines) and (xii) pyloric ceaca. All counts were made on fixed material following the methodology in Pravdin (1966) with the following modifications: vertebrae count does not include the urostyle; gill raker and lateral line pore counts are all-inclusive aided by a × 8–16 binocular microscope; the last branched ray in the dorsal and anal fins, which looks like two separate rays, is counted as one. A description and body position of the 28 morphometric measurements are described in Fig. 1. To control for allometric effects all morphometric measurements were transformed into a per cent of the standard length (L_S) prior to all analyses. Means, S.D. and minimum and maximum values for all measurements and counts are reported. Uni-variate statistical differences between these values for the two taxa were assessed with a Mann–Whitney *U*-test using the SPSS software package.

A principal component analysis (PCA) was performed on the covariance matrix of morphometric and meristic characters separately, and all principal components with eigenvalues >1 were saved as new variables. These variables were then used as input for a canonical discriminant analysis (CDA) to quantify the discriminatory power of these factors (for meristic and morphometric separately) in delineating the two taxa. Prior probabilities of inclusion were considered equal for both groups.

RESULTS

MTDNA

Length variation of the mtDNA control region was observed within *S. ohridanus*. This variation was due to an 82 bp repeat unit in the 3′ region. There were one to three copies of this repeat in addition to a shorter homologous unit of 66 bp at the 5′ end of the repeat region in all samples (Table II). Higher

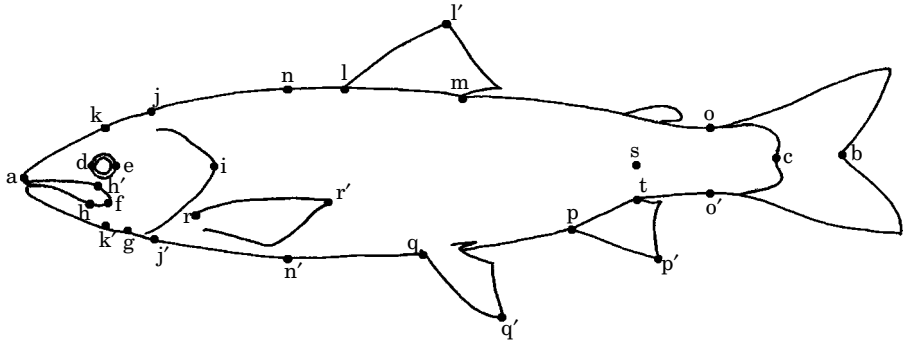

FIG. 1. Position and description of the 28 morphometric characteristics based on the lettered landmark positions: ac, length up to the end of the lateral line scales; ic, trunk length; ad, snout length; de, horizontal eye diameter; ei, postorbital part of head; ai, head length; jj', head length at occiput; kk', head length at eye; forehead width (landmark position not seen on figure); af, length of upper jaw; hh', width of upper jaw; ag, length of lower jaw; nn', the greatest body depth; oo', the least body depth, body thickness (landmark position not seen on figure); al, antedorsal distance; mc, post-dorsal distance; ap, anteanal distance; aq, anteventral distance; sc, caudal peduncle length; rq, pectoventral distance; qp, ventroanal distance; lm, length of dorsal fin base; ll', depth of anterior part of dorsal fin; pt, length of anal fin base; pp', anal fin depth; rr', pectoral fin length; qq', ventral fin length.

TABLE II. Variable nucleotide positions in the repeats at the 3′-end of the *Salmo ohridanus* and *Salmo trutta* mtDNA control region and the observed frequency of each repeat. Repeats are counted from the 3′ to 5′ end

		1	3	4	6	8	12	15	18	20	22	28	30	32	34	35	39	44	45	48	53	54	57	69–71	72	73–84	*S. ohridanus*	Lake Ohrid brown trout (this study)	Lake Ohrid brown trout (Sell & Spirkovski, 2004)
First repeat	A	G	C	G	T	A	C	/	G	G	A	T	A	T	T	T	A	T	T	G	/	T	T	CTT	T	AATTAT AAAAGT	1	/	/
	B										C											G	C				1	/	/
	C							A			C											G	C				1	/	/
	D		T					A			C											G	C				1	/	/
	E		T								C												C				2	/	/
	F		T			/					C						C					G	C				1	/	/
	G		T								C											G	C				21	/	/
	H		T								C											G	C		C		1	/	/
	I*		T								C				A	G					A	G					1	/	/
	J		T								C										A	G	C				/	/	1
	K		T								C									A	A	G	C				/	/	1
	L		T								C										A	G					/	24	3
	M		T								C							/	G	A	A	G	C				/	/	1
	N		T								C	C			C			/	G	A	A	G	C				/	/	1
	Ad1										C										A	G							
	Ma1		A								C											G							
	Me1										C		G									G							
	Da1									A	C											G							
	At1										C		G																
	S.ob.			A							C											G							
Second, third or fourth repeat	O		T		C		T		T		C	C			C			/	G	A	A	G	C				26	/	9
	P		T		C		T		T		C	C		G	C			/	G	A	A	G	C				1	/	/
	Q		T		C		T		T		C	C			C	C		/	G	A	A	G	C				1	/	/
	R		T		C				T		C	C			C			/	G	A	A	G	C				1	/	/
Last repeat (shorter)	S		T		C		T		T		C	C			C			/	G	A	A	G	C	///	/	////////////	6	18	6
	T				C		T		T		C	C			C			/	G	A	A	G	C	///	/	////////////	1	6	1
	U		T		C				T		C	C			C			/	G	A	A	G	C	///	/	////////////	20	/	/
	V	/	T		C				T		C	C			C			/	G	A	A	G	C	///	/	////////////	1	/	/

(Ad1, Ma1, Me1, Da1 and At1, haplotypes of *S. trutta*; accession numbers are listed in Table IV; *, hybrid individual; *S. ob.*, *Salmo obtusirostris*);/, one base pair deletion.

homology was observed among the first repeats in different *S. ohridanus* CR haplotypes than between the first and subsequent repeats within the same CR sequence (Table II). The 66 bp sequence of the shorter units were identical or very similar to the analogous base pairs in the other repeat units (Table II). In all samples of Lake Ohrid brown trout, only one copy of the 82 bp repeat and the shorter unit was found.

Excluding the repeat units, the final alignment for all 54 individuals was 921 bp. There were a total of 15 haplotypes based on 21 variable sites, 17 of which were parsimony informative (Table III). The transition: transversion ratio for the combined data set of *S. ohridanus* and Lake Ohrid brown trout was 2·6, whereas it was 7 : 1 within *S. ohridanus*, and no transversions were found among Lake Ohrid brown trout haplotypes. Twelve haplotypes were found in *S. ohridanus*, and four in Lake Ohrid brown trout. Phylogenetic analysis revealed that all but one of the *S. ohridanus* haplotypes clustered into a well-supported clade, containing haplotypes that were 0·9 to 1·4% divergent from those found in Lake Ohrid brown trout (Fig. 2). Within this clade, the 11 haplotypes differed by one to four nucleotides (pairwise distance of 0·1–0·4 %). The remaining haplotype was shared between *S. ohridanus* (Haplo15; $n = 1$) and Lake Ohrid brown trout ($n = 2$; Table III). This haplotype differed from the common Adriatic (Ad1, AY260516) haplotype of brown trout by four transitions, two of which were detected in first 310 bp analysed in Sell & Spirkovski (2004), and therefore, most probably represents haplotype Ad-s10 described therein. The remaining three haplotypes found in Lake Ohrid brown trout differed from the Ad1 haplotype by one to seven mutations (0·1 to 0·8%; Table III).

For the cytochome *b* gene, a total of 295 bp were sequenced, and two haplotypes were resolved, one corresponding to each of the two taxon analysed, and both representing previously reported haplotypes in *S. ohridanus* (accession number AF053590; Phillips *et al.*, 2000) and brown trout (accession number U63892, Apostolidis *et al.*, 1997).

PHYLOGENETIC ANALYSES

Exploratory phylogenetic analysis using the entire CR revealed evidence of a high degree of homoplasy, or other mechanism responsible for confounding the phylogenetic signal in the 3′ end of the CR, especially the repeat region, and perhaps some adjacent sites as well. In the first repeat there were no synapomorphic sites relating to the differentiation between *S. trutta* (including Lake Ohrid brown trout) and either *S. ohridanus* or *S. obtusirostris*; in subsequent repeats there was little variation and no correspondence to taxa (Table II). For this reason, phylogenetic analysis was limited to the 5′ end of the control region (480 bp) together with the 295 bp-long cyt b gene fragment. For all reconstruction approaches (NJ, MP and ML) there was high support for one clade containing *S. ohridanus* and *S. obtusirostris* and the relationship of this clade was reciprocally monophyletic to brown trout using the NJ and MP approaches, while maximum likelihood displayed a polychotomy for brown trout (Fig. 2). Regardless of the approach, haplotypes from Lake Ohrid brown trout appeared as an unsupported cluster most closely related to other brown trout haplotypes typically found in the Adriatic basin.

TABLE III. Variable nucleotide positions in *Salmo ohridanus* and Lake Ohrid brown trout haplotypes, found in the first 921 bp of the mitochondrial DNA control region and the frequency of each haplotype in each taxon

		15	42	70	161	241	271	277	353	401	402	412	418	488	545	563	678	752	766	910	919	921	bel	let
S. ohridanus	Haplo1	T	T	A	G	T	C	G	T	A	A	C	C	G	T	C	T	–	C	G	A	C	12	/
	Haplo2	C																	T	A			1	/
	Haplo3				A					G										A			1	/
	Haplo4					C														A			1	/
	Haplo5									G													1	/
	Haplo6											A											7	/
	Haplo7												T										2	/
	Haplo8																C						1	/
	Haplo9																		T	A			1	/
	Haplo10																			A			2	/
	Haplo11																			A		/	1	/
Lake Ohrid brown trout	Haplo12		C			C	A	C	A		G			A	C				T	A			/	20
	Haplo13		C			C	A	C	A		G			A	C	T			T	A			/	1
	Haplo14		C	G		C	A	C	A		G			A	C			C	T	A	G		/	1
	Haplo15		C	G	A	C	A	C	A		G			G	C	T			C	A	G		1	2

bel, *Salmo ohridanus*; let, Lake Ohrid brown trout;/, one base pair deletion.

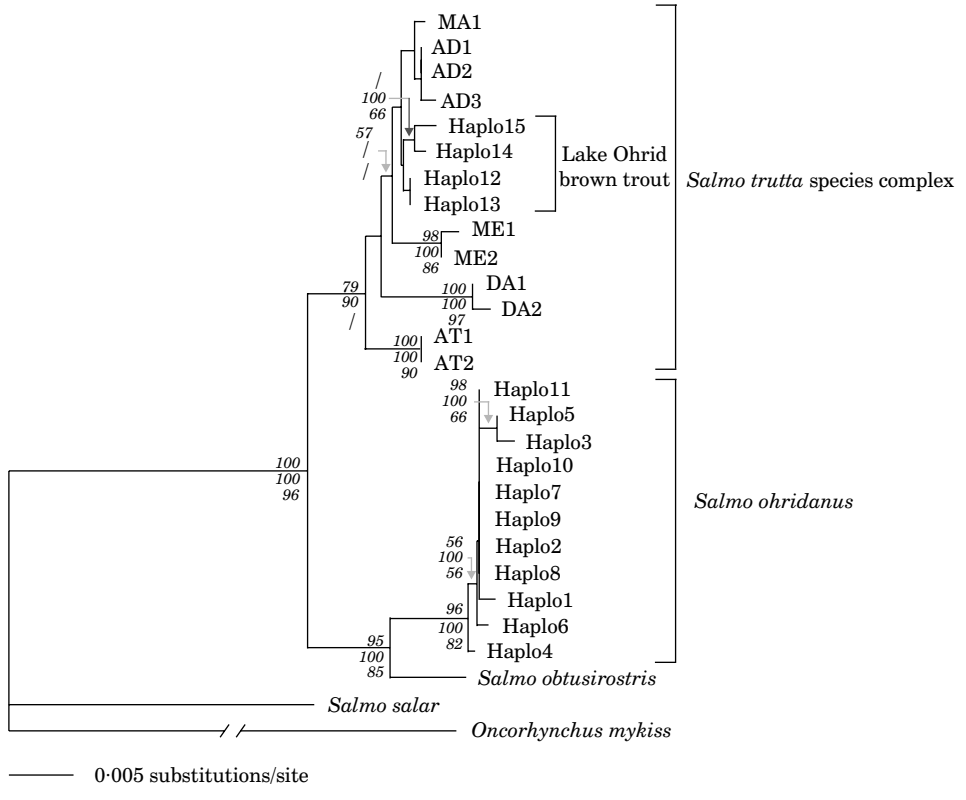

FIG. 2. Neighbour-joining (NJ) tree for *Salmo* based on 480 bp of 5′-end mtDNA control region and 295 bp of cytochrome *b* gene, based on a Kimura 2-parameter substitution model. Bootstrap support values from top to bottom refer to NJ, maximum parsimony and maximum likelihood (HKY + I + G model, transition: transversion = 4·0465; $\alpha = 0{\cdot}6782$; per cent invariable sites = 0·6643) methods. 1, values <50.

MICROSATELLITES

All individual loci were in HWE for *S. ohridanus* and five of seven for Lake Ohrid brown trout after correcting for multiple tests (Table IV). In Lake Ohrid brown trout, there was a borderline significant departure from HWE across all loci. For the loci in Lake Ohrid brown trout exhibiting lower observed than expected heterozygosity, the expected frequency of null alleles ranged from 2·3 (Strutta58) to 7 (BFR0001) %. One locus combination (Ssa85 + Struta58) in *S. ohridanus* (in Lake Ohrid brown trout Ssa85 was monomorphic) revealed significant departure from linkage equilibrium after correction for multiple tests ($P = 0{\cdot}005$). Four *S. ohridanus* individuals showed a duplicated pattern at four loci (*Ssa197*, *OMM1064*, *Ssosl417* and Struta58). The duplicated pattern (three or four alleles) was detected on all four loci in each of these four individuals. These four individuals were removed from further microsatellite analyses.

The mean number of alleles (*A*) in *S. ohridanus* was 7·71 (4–15), and for Lake Ohrid brown trout 10·28 (1–18). Average expected heterozygosity in both taxa was high; 0·5820 for *S. ohridanus* and 0·6781 for Lake Ohrid brown trout. Highly distinct

allelic profiles were observed for *S. ohridanus* and Lake Ohrid brown trout across all loci analysed (Fig. 3), reflected in highly significant F_{st} (0·1517 to 0·5513, Table V) or R_{st} (0·579 to 0·895) values between the taxa. One *S. ohridanus* individual, however, had 'Lake Ohrid brown trout specific' alleles at five microsatellite loci.

MORPHOLOGY

Nine of the 12 meristic characters were significantly different between taxa ($P < 0·05$; Table VI), five of which (total dorsal rays, branched pectoral rays, gill rakers, vertebrae and pyloric caeca) were highly significant ($P < 0·001$). No meristic, however, character displayed non-overlapping ranges. Of the 28 morphometric characters, 21 showed significant differences between taxa, 14 of which were highly significant (Table VII). Of these, four characters revealed

TABLE IV. Number of alleles (A), expected and observed heterozygosity, and F_{is} values for the two analysed taxa. N, the number of individuals genotyped in each taxon

	Salmo ohridanus	Lake Ohrid brown trout		*S. ohridanus*	Lake Ohrid brown trout
Ssosl438			*Ssa85*		
N	26	24	N	26	24
A	4	13	A	4	1
H_{exp}	0·2277	0·8663	H_{exp}	0·618	0
H_{obs}	0·25	0·7917	H_{obs}	0·6429	0
F_{is}	−0·08	0·1073*	F_{is}	−0·0221	
Ssa197			*OMM1064*		
N	26	24	N	26	24
A	8	12	A	6	18
H_{exp}	0·6996	0·8429	H_{exp}	0·6084	0·9036
H_{obs}	0·7143	0·7917	H_{obs}	0·6296	0·9583
F_{is}	−0·0028	0·0819	F_{is}	−0·0161	−0·0393
Ssosl417			*BFRO001*		
N	26	24	N	26	23
A	15	6	A	4	10
H_{exp}	0·8909	0·4783	H_{exp}	0·2532	0·776
H_{obs}	0·9286	0·5417	H_{obs}	0·2143	0·6522
F_{is}	−0·0241	−0·1115	F_{is}	0·1714	0·1811*
Struta58					
N	26	24			
A	13	12			
H_{exp}	0·7761	0·8793			
H_{obs}	0·8571	0·875			
F_{is}	−0·0863	0·0262			
A	7,71	10,28			
H_{exp}	0·5820	0·6781			
H_{obs}	0·6053	0·6586			
F_{is}	−0·0217	0·0501*			

(*, significant at $P < 0·05$ after correction for multiple tests).

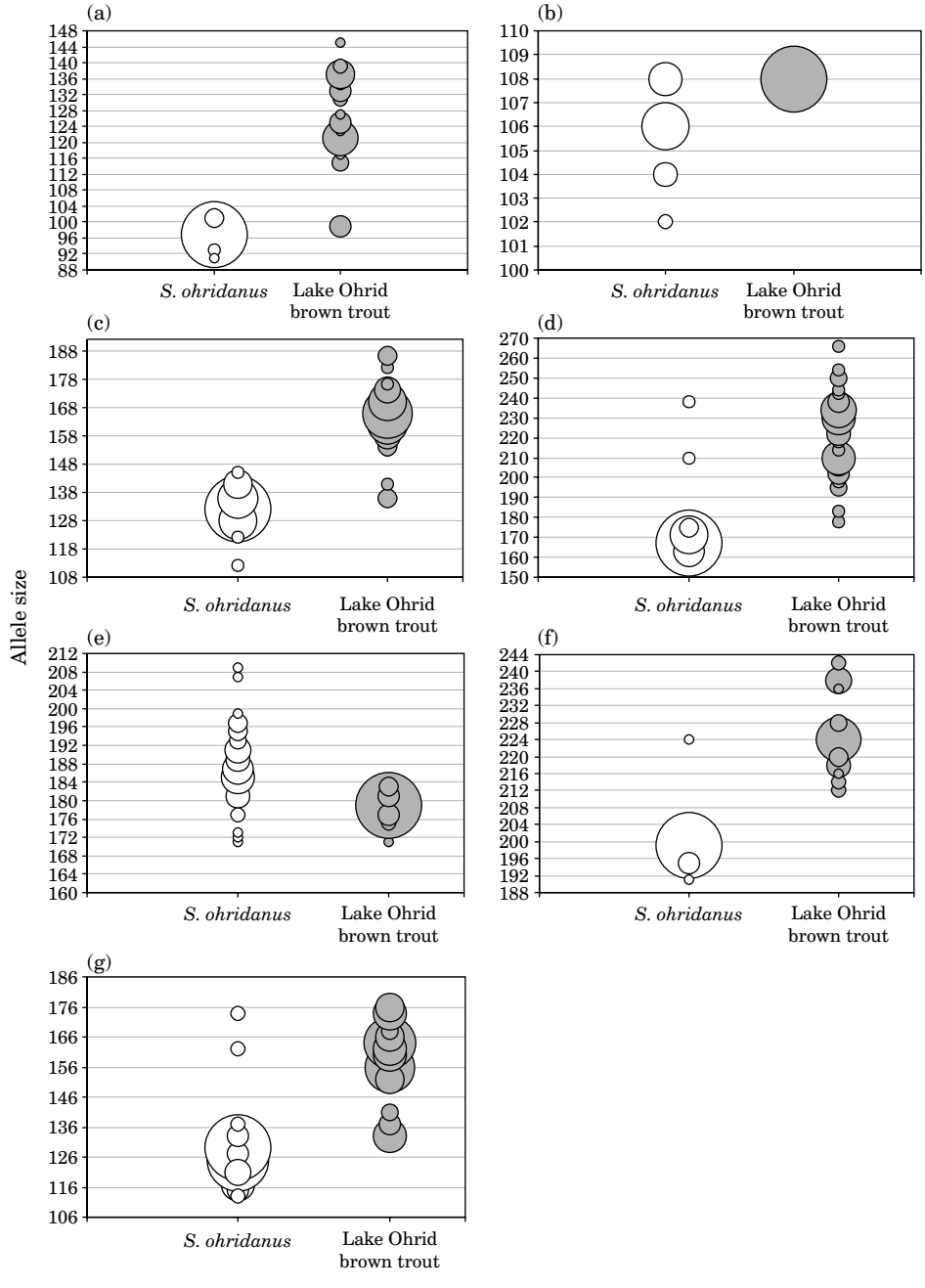

FIG. 3. Allele frequencies and size distributions of seven microsatellite loci: (a) *SSOSL438*, (b) *Ssa85*, (c) *Ssa197*, (d) *OMM1064*, (e) *SSOSL417*, (f) *BFRO001* and (g) *Strutta 58* in *Salmo ohridanus* and Lake Ohrid brown trout. The areas correspond to the frequencies of the respective alleles in each taxon.

TABLE V. Locus specific and overall R_{st} and pairwise F_{st} values

Locus	R_{st}	F_{st}
Ssosl438	0·793	0·4532
Ssa85	0·581	0·5513
Ssa197	0·895	0·2068
OMM106	0·848	0·2285
Ssosl417	0·579	0·2890
BFRO001	0·873	0·4823
Struta58	0·774	0·1517
Overall	0·8149	0·32930

non-overlapping ranges (snout length, horizontal eye diameter, postorbital part of head and length of lower jaw).

A PCA on the 28 morphometric characters extracted eight factors explaining 84% of the variance in the data, while analysis of the 12 meristic characters extracted five factors explaining 72% of the variance in the data. A bi-variate scatter plot of the first principal component of each analysis revealed highly distinct groups representing the two taxa (Fig. 4). Based on the CDA using components from the morphometric PCA, all individuals could be assigned to their respective taxon with a 100% probability of inclusion. In the CDA based on components from the meristic PCA, however, two *S. ohridanus* individuals were misclassified, although probabilities of inclusion were still quite high (mean $\pm$ S.D. for *S. ohridanus* 93·6 $\pm$ 22·0%; Lake Ohrid brown trout 99·9 $\pm$ 0·2%). Removal of these two misclassified individuals did not alter the results with respect to the lack of non-overlapping meristic characters, although the individual probability of inclusion was raised to 99·1 $\pm$ 2·8%.

DISCUSSION

All genetic data from the mitochondrial and nuclear genome as well as the morphological data (morphometric and meristic characters) revealed an unambiguous distinction between *S. ohridanus* and Lake Ohrid brown trout, both from Lake Ohrid, Macedonia. Furthermore, phylogenetic analysis incorporating 30 *S. ohridanus* individuals is congruent with results based on the same two individuals (Phillips *et al.*, 2000; Snoj *et al.*, 2002), in placing *S. ohridanus* as a sister taxon to *S. obtusirostris*, and grouping these two taxa in a clade that reveals a sister-clade status to the *S. trutta* complex. However, unambiguous evidence of hybridization and introgression, however, is seen in one, phenotypically characterized *S. ohridanus* that carries a typical brown trout haplotype (Haplo15), as well as a "mixture" of taxon-specific microsatellite alleles. Whether or not this evidence of hybridization stems from natural gene flow, or escapees from a cross-breeding experiment is impossible to determine. Successful artificial hybridization between female *S. ohridanus* and male Lake Ohrid brown trout has been reported (Rakaj & Flloko, 1995).

Another highly unique molecular character in *S. ohridanus* was the 82 bp repeat region in the 3' end of the CR, first reported in *Salmo* by Sell &

TABLE VI. Number of individuals, means ± S.D. and ranges for the 12 meristic characters analysed in the two taxa. Results of Mann–Whitney *U*-test (*Z* statistic and *P*-value) between the two taxa for each of 12 meristic characters are also given

	Taxon					
	S. ohridanus ($n = 33$)		Lake Ohrid brown trout ($n = 16$)		Mann–Whitney *U*	
	Mean ± S.D.	Range	Mean ± S.D.	Range	*Z*	*P*
(i) Lateral line pores	106·67 ± 3·35	102·00–116·00	109·19 ± 3·54	105·00–116·00	−2·124	0·034
(ii) Unbranched dorsal rays	3·70 ± 0·59	3·00–5·00	4·25 ± 0·58	3·00–5·00	−2·840	0·005
(iii) Branched dorsal rays	8·55 ± 0·56	8·00–10·00	9·19 ± 0·54	8·00–10·00	−3·310	0·001
(iv) Total dorsal rays	12·21 ± 0·82	11·00–14·00	13·50 ± 0·82	12·00–15·00	−4·170	<0·001
(v) Branched pectoral rays	11·36 ± 0·55	11·00–13·00	12·06 ± 0·57	11·00–13·00	−3·627	<0·001
(vi) Ventral rays	8·09 ± 0·29	8·00–9·00	8·00 ± 0·00	8·00–8·00	−1·232	0·218
(vii) Unbranched anal rays	3·61 ± 0·56	3·00–5·00	3·94 ± 0·57	3·00–5·00	−1·845	0·065
(viii) Branched anal rays	8·00 ± 0·61	7·00–9·00	8·19 ± 0·54	7·00–9·00	−1·024	0·306
(ix) Gill rakers	19·21 ± 1·11	17·00–21·00	21·56 ± 1·63	18·00–24·00	−4·446	<0·001
(x) Branchiostegal rays	10·12 ± 0·48	9·00–11·00	9·69 ± 0·60	9·00–11·00	−2·561	0·010
(xi) Vertebrae	48·52 ± 1·39	46·00–52·00	50·13 ± 0·89	49·00–52·00	−3·927	<0·001
(xii) Pyloric caeca	41·24 ± 7·41	28·00–61·00	62·94 ± 4·78	55·00–74·00	−5·496	<0·001

TABLE VII. Number of individuals, means ± S.D. and ranges for the 28 morphometric characters (see Fig. 1) analysed in the two taxa. All values are given as a per cent of the standard length. Results of the Mann–Whitney *U*-test (*Z* statistic and *P*-value) between the two taxa for each character are also given

	Taxon					
	Salmo ohridanus ($n = 33$)		Lake Ohrid brown trout ($n = 16$)		Mann–Whitney *U*	
	Mean ± S.D.	Range	Mean ± S.D.	Range	*Z*	*P*
ac – Length up to scales	94·34 ± 0·70	93·17–96·17	92·60 ± 0·57	91·47–93·50	−5·437	<0·001
ic – Trunk length	75·92 ± 1·55	72·59–79·50	74·11 ± 1·36	71·88–76·43	−3·518	<0·001
ad – Snout length	6·16 ± 0·38	4·90–6·70	7·45 ± 0·33	6·92–8·19	−5·629	<0·001
de – Horizontal eye diameter	5·38 ± 0·43	4·63–6·48	3·80 ± 0·34	3·24–4·66	−5·608	<0·001
ei – Postorbital part of head	10·07 ± 0·37	9·37–10·76	11·51 ± 0·57	10·73–12·69	−5·608	<0·001
ai – Head length	20·84 ± 0·82	19·28–22·63	22·16 ± 1·05	20·72–24·60	−4·040	<0·001
jj' – Head length at occiput	15·08 ± 0·59	13·90–16·58	15·41 ± 0·67	14·15–16·80	−1·652	0·098
kk' – Head length at eye	10·68 ± 0·57	9·61–11·73	11·15 ± 0·47	10·13–11·95	−2·719	0·007
Forehead width	6·27 ± 0·31	5·45–6·82	6·80 ± 0·37	5·93–7·38	−4·233	0·000
af – Length of upper jaw	6·97 ± 0·44	6·23–7·77	8·75 ± 0·66	7·18–9·91	−5·330	<0·001
hh' – Width of upper jaw	2·18 ± 0·21	1·78–2·57	2·33 ± 0·19	2·02–2·75	−2·154	0·031
ag – Length of lower jaw	11·32 ± 0·65	9·79–12·51	13·89 ± 0·74	12·75–15·77	−5·629	<0·001
nn' – Greatest body depth	19·27 ± 1·65	16·57–24·52	20·45 ± 1·02	18·01–21·97	−3·102	0·002
oo' – Least body depth	7·44 ± 0·37	6·55–8·39	8·51 ± 0·32	7·78–9·09	−5·427	<0·001
Body thickness	11·61 ± 0·88	10·11–13·26	10·68 ± 0·62	9·60–11·82	−3·369	0·001
al – Antedorsal distance	42·38 ± 1·70	39·57–47·48	42·24 ± 1·36	40·40–45·81	−0·554	0·579
mc – Postdorsal distance	42·20 ± 1·60	38·90–46·51	39·85 ± 1·11	37·95–41·33	−4·424	<0·001

TABLE VII. Continued

	Taxon					
	Salmo ohridanus ($n = 33$)		Lake Ohrid brown trout ($n = 16$)		Mann–Whitney U	
	Mean ± S.D.	Range	Mean ± S.D.	Range	Z	P
ap – Anteanal distance	69·05 ± 1·40	64·27–71·85	68·18 ± 1·67	64·78–70·64	−1·791	0·073
aq – Anteventral distance	49·98 ± 1·47	46·41–53·00	49·50 ± 1·29	47·07–51·95	−1·343	0·179
sc – Caudal peduncle length	17·59 ± 0·93	15·50–19·58	17·22 ± 0·79	15·74–18·29	−1·429	0·153
rq – Pectoventral distance	31·48 ± 1·42	29·11–34·11	29·83 ± 1·23	26·69–31·45	−3·326	0·001
op – Ventroanal distance	20·13 ± 1·40	17·40–22·89	19·24 ± 1·01	17·15–21·13	−2·228	0·026
lm – Length of dorsal fin base	10·31 ± 0·74	8·97–11·55	12·38 ± 0·65	11·07–13·66	−5·458	<0·001
ll' – Depth of anterior part of dorsal fin	13·76 ± 1·03	11·25–16·01	14·28 ± 0·94	12·88–15·93	−1·695	0·090
pt – Length of anal fin base	8·62 ± 0·63	7·37–9·76	8·91 ± 0·47	8·12–9·69	−1·397	0·163
pp' – Anal fin depth	10·93 ± 0·73	9·59–12·54	13·00 ± 1·46	8·90–14·90	−4·444	<0·001
rr' – Pectoral fin length	16·41 ± 0·84	14·18–17·63	17·03 ± 1·07	14·59–18·83	−2·196	0·028
qq' – Ventral fin length	11·79 ± 0·67	10·13–13·16	13·20 ± 0·83	11·35–14·26	−4·563	<0·001

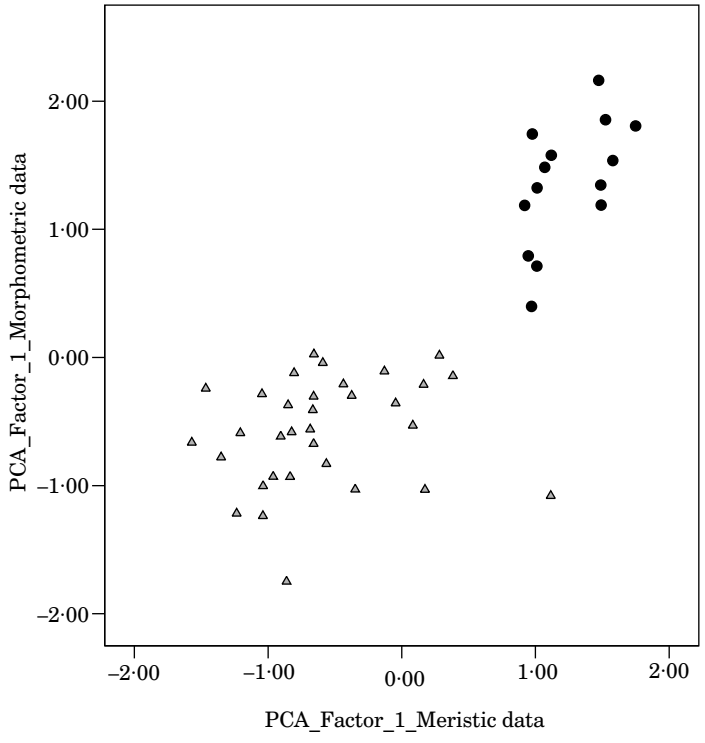

FIG. 4. Scatterplot of the first principal component derived from PCA of 12 meristic characters, and the first principal component derived from PCA of 28 morphometric characters (standardized to per cent of standard length) in *Salmo ohridanus* (△) and Lake Ohrid brown trout (●).

Spirkovski (2004). A similar analogous repeat has been reported in *Thymallus* (Weiss *et al*., 2002) and *Coregonus* (Brzuzan, 2000), and this repeat has also been found in the genus *Hucho* and *Brachymystax* (Froufe., unpubl. data). The phylogenetic signal within the first copy of this repeat conflicts with the expectation that the *S. trutta* complex is monophyletic with respect to the two other *Salmo* species, *S. ohridanus* and *S. obtusirostris* (Phillips *et al*., 2000, 2004; Snoj *et al*., 2002). Furthermore, the pattern of substitution became increasingly blurred when subsequent repeats were analysed. Parallel character-state changes in such repeats are not unusual and if they appear in a non-random pattern, they imply a common mechanism (Broughton *et al*., 1998). As recombination in animal mtDNA is very rare, repeats are generally thought to be the result of intramolecular processes (Buroker *et al*., 1990), and could occur in systems where specific secondary structures have the potential to interfere with DNA replication. The mechanisms evoked to generate variation in mtDNA repeats have included slip-strand mispairing during replication (Buroker *et al*., 1990; Broughton & Dowling, 1997; Savolainen *et al*., 2000; Mundy & Helbig, 2004), recombination and transposition (Rand & Harrison, 1989) and unequal crossing-over or gene conversion (Hoelzel *et al*., 1993, 1994). Each of these mechanisms has been shown to fit a particular data set but a common mechanism has not been confirmed.

In Lake Ohrid brown trout, there was no length variation in the repeat region (one repeat plus one incomplete repeat; Table II). Sell & Spirkovski (2004), however, reported four length variants, involving at least 10 haplotypes, although seven of these involved substitutions in the repeat region in individuals that displayed a monomorphic RFLP pattern across three mtDNA regions (CR, NADH-1 and NADH-3/4). In fact, the differentiation reported in the so-called summer form (known locally as *S. letnica aestivalis*) was based on the occurrence of a single haplotype (at *c.* 50% frequency) variant in this apparently unstable repeat region (Sell & Spirkowski, 2004). Samples of the summer form of Lake Ohrid brown trout are not available (sampling has been restricted on the lake since early in 2004), but the reliability of any phylogenetic or population genetic inferences based solely on differentiation found in this repeat, considering the present observations as well as the concerns raised above, should be questioned.

Despite the detection of a single hybrid, microsatellite analysis did not reveal evidence of contemporary gene flow between these taxa. While both F_{st} and R_{st} values where highly significant, R_{st} values, reflecting an evolutionary distance measure based on disparate allele sizes, were especially high and relatively uniform among loci. As all loci for *S. ohridanus* were in HWE, the sample appears to stem from a single population, with no substructure. While there was significant linkage for loci *Ssa85* and *Strutta58*, both loci are mapped on the *S. trutta* genome and appear on different linkage groups (*Ssa85* on BT4 and *Strutta* on BT26; Gharbi, 2001). Potential mechanisms that could produce such a result include pseudo-linkage of co-adapted genes, or real physical linkage *via* a chromosomal rearrangement in *S. ohridanus* with respect to *S. trutta.*

The two loci that displayed a significant departure from HWE in Lake Ohrid brown trout may have contained null alleles. For one locus (*BRF0001*), displaying the highest estimate of null alleles (7%), one individual did not amplify, which could also be interpreted as evidence of a null allele in the form of a null homozygote. Given the reported population structure of Lake Ohrid brown trout, however, the relatively small differences between observed and expected heterozygosities could also result from a slight Wahlund effect. More importantly, the differentiation indices between taxa are consistent across all loci, and no evidence of null alleles was seen for *S. ohridanus*. More extensive sampling and typing of Lake Ohrid brown trout will be necessary to draw further inferences on the potential for a population subdivision.

Within *S. ohridanus* samples, duplication was observed in four individuals at four loci. Duplicated loci in salmonids are common, due to the incomplete diploidization of this autotetraploid lineage, and anueploidy and partial genomic duplication has also been reported (Allendorf & Thorgaard, 1984). It is not clear, however, what mechanism is responsible for the duplication of four common loci (out of seven typed), in some ($n = 4$), but not all individuals in a population.

Morphological analysis of *S. ohridanus* reveals that this taxon is highly divergent from Lake Ohrid brown trout. This difference appears larger for morphometric as opposed to meristic characters, as the latter data set revealed no non-overlapping characters. Presumably, this reflects selection on body form related to its highly distinct deep-water niche, whereas there has been either

insufficient time (*via* random mechanisms) or insufficient selective pressure (*i.e.* deterministic mechanisms) to promote similar levels of divergence in meristic characters.

As the mtDNA (CR)-based divergence between *S. ohridanus* and the *S. trutta* complex (0·9–1·9%) is equal or less than the total divergence within *S. trutta* (1·21 to 2·19% net divergence among five mtDNA-defined lineages, Bernatchez, 2001), it might appear that morphological evolution in *S. ohridanus* has been extraordinarily fast for a *Salmo* taxon. Pairwise divergence estimates between *S. ohridanus* and *S. trutta* based on the cytochrome *b* gene (cyt b) are 2·6 to 4·2%, however, considerably greater than the divergence of cyt b within *S. trutta* (0 to 1·4%; calculated from GenBank sequences, Table I). The reason for this discrepancy between interspecific divergences based on the CR and cyt b genes appears to be saturation (*i.e.* evolutionary constraints) in the CR within the genus *Salmo*, an observation additionally supported by the low transition: transversion ratio overall (2·6), compared to within taxon ratios. Species-specific constraints on the CR, which lead to underestimated interspecific divergences compared to coding segments have been clearly shown in the salmonid genera *Thymallus* (Froufe *et al.*, 2005*a*) and *Hucho* (Froufe *et al.*, 2005*b*), a phenomena that is not limited to fishes (Roukonen & Kvist, 2002).

Thus, the actual divergence between the *S. ohridanus* and *S. obtusirostris* clade and *S. trutta* must be considerably greater than phylogenetic analyses based on, or including the CR. For example, if it is assumed that the *S. ohridanus* and *S. obtusirostris* lineage and *S. trutta* diverged from a common ancestor and that the *S. trutta* complex is at least 2 million years old (Osinov & Bernatchez, 1996; Apostolidis *et al.*, 1997; Antunes *et al.*, 2002), an approximation of the age of the *S. ohridanus* and *S. obtusirostris* lineage can be derived. As the net divergence among clades within *S. trutta* for the cyt b gene is 1·4%, and the net divergence between *S. trutta* and the *S. ohridanus* and *S. obtusirostris* clade is 2·9%, then an age for this split can be roughly calculated to be at least 4·2 million years, above the lower boundary of the age of Lake Ohrid (4–10 million years; Bănărescu, 1991).

While it must be assumed that *S. ohridanus* evolved in Lake Ohrid, adapting to a deepwater niche, without fossil evidence it is impossible to date its colonization or place a time frame around its morphological divergence. Considering that there is no record of *S. obtusirostris* in the lake, one possible scenario involves a lake residence of *S. ohridanus* roughly equalling the age of the split between the two taxa (*c.* 1 million years ago). This would imply a relatively rapid evolution of *S. ohridanus* morphology relative to the extant variation in the genus *Salmo*. An alternative scenario involving the existence of the common ancestor of *S. ohridanus* and *S. obtusirostris* in the lake, however, cannot be excluded. Further comparison of *S. ohridanus* with the extant sub-specific variation known in *S. obtusirostris*, as well as demographic analysis of an increased sample size of *S. ohridanus* might uncover a signature of recent expansion, and population differentiation. This could lend support to estimates of the age of the taxon in the lake. At present, it can only be concluded that the age of *S. ohridanus* in Lake Ohrid lies between the estimated divergence times for the split between the *S. ohridanus* and *S. obtusirostris* and *S. trutta* clades (at maximum), and the split between *S. ohridanus* and *S. obtusirostris* (at minimum).

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References

Allendorf, F. W. & Thorgaard, G. H. (1984). Tetraploidy and the evolution of salmonid fishes. In *Evolutionary Genetics of Fishes* (Turner, B. J., ed.), pp. 23–24. New York and London: Plenum Press.

Antunes, A., Templeton, A. R., Guyomard, R. & Alexandrino P. (2002). The role of nuclear genes in intraspecific evolutionary inference: genealogy of the transferrin gene in the brown trout. *Molecular Biology and Evolution* **19,** 1272–1287.

Apostolidis, A. P., Triantaphyllidis, C., Kouvatsi, A. & Economidis P. S. (1997). Mitochondrial DNA sequence variation and phylogeography among *Salmo trutta* L. (Greek brown trout) populations. *Molecular Ecology* **6,** 531–542.

Bănărescu, P. (1991). *Zoogeography of Fresh Waters*, Vol 2. Wiesbaden: AULA-Verlag.

Bănărescu, P. (2004). Distribution pattern of the aquatic fauna of the Balkan peninsula. In *Balkan Biodiversity, Pattern and Process in the European Hotspot* (Griffiths H. I. & Kryštufek B., eds), pp. 203–217. Dordrecht, Boston and London: Kluwer Academic Publishers.

Belkhir, K., Borsa, P., Chikhi, L., Raufaste, N. & Bonhomme, F. (1996–2004). *GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations*. Montpellier: *Laboratoire Génome, Populations, Interactions, CNRS UMR 5000, Université de Montpellier II*.

Berg, L. S. (1910). Ibidem 15, 2–3 (citat nach Berg 1932: Übersicht der Verbretung der Süsswasserfische Europas. *Zoogeographica* **1,** 107–208.

Bernatchez, L. (2001). The evolutionary history of brown trout (*Salmo trutta* L.) inferred from phylogeographic, nested clade, and mismatch analysis 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* Mitchill). *Molecular Biology and Evolution* **10,** 1002–1014.

Bernatchez, L., Guyomard, R. & Bonhomme, F. (1992). DNA sequence variation of the mitochondrial control region among geographically and morphologically remote European brown trout (*Salmo trutta*) populations. *Molecular Ecology* **1,** 161–173.

Bianco, P. G. (1990). Potential role of the palaeohistory of the Mediterranean and Paratethys basin on the early dispersal of Euro-Mediterranean freshwater fishes. *Ichthyological Exploration of Freshwaters* **1,** 167–184.

Brookfield, J. F. Y. (1996). A simple new method for estimating null allele frequency from heterozygote deficiency. *Molecular Ecology* **5,** 453–455.

Broughton, R. E. & Dowling, T. E. (1997). Evolutionary dynamics of tandem repeats in the mitochondrial DNA control region of the minnow *Cyprinella spiloptera*. *Molecular Biology and Evolution* **14,** 1187–1196.

Broughton, R. E., Naylor, G. J. P. & Dowling, T. E. (1998). Conflicting phylogenetic patterns caused by molecular mechanisms in mitochondrial DNA sequences. *Systematic Biology* **47,** 696–701.

Brzuzan, P. (2000). Tandemly repeated sequences in mtDNA control region of whitefish, *Coregonus lavaretus*. *Genome* **43,** 584–587.

Buroker, N. E., Brown, J. R., Gilbert, T. A., O'Hara, P. J., Beckenbach, A. T., Thomas, W. K. & Smith, M. J. (1990). Length heteroplasmy of sturgeon mitochondrial DNA; an illegitimate elongation model. *Genetics* **124,** 157–163.

Frogley, M. R. & Preece, R. C. (2004). A faunistic review of the modern and fossil molluscan fauna from Lake Pamvotis, Ioannina, an ancient lake in NW Greece: implications for endemism in the Balkans. In *Balkan Biodiversity, Pattern and Process in the European Hotspot* (Griffiths H. I. & Kryštufek B., eds), pp. 243–260. Dordrecht, Boston and London: Kluwer Academic Publishers.

Froufe, E., Knizhin, I. & Weiss, S. (2005*a*). Phylogenetic analysis of the genus *Thymallus* (grayling) based on mtDNA control region and ATPase 6 genes, with inferences on control region constraints and broad-scale Eurasian phylogeography. *Molecular Phylogenetics and Evolution* **34,** 106–117.

Froufe, E., Alekseev, S., Knizhin, I. & Weiss, S. (2005*b*). Comparative mtDNA sequence (control region, ATPase 6 and NADH-1) divergence in *Hucho taimen* (Pallas) across four Siberian river basins. *Journal of Fish Biology* **67,** 1040–1053.

Goodman, S. J. (1997). Rst Calc: a collection of computer programs for calculating estimates of genetic differentiation from microsatellite data and a determining their significance. *Molecular Ecology* **6,** 881–885.

Griffiths, H. I. & Frogley, M. R. (2004). Fossil ostracods, faunistics and the evolution of regional biodiversity. In *Balkan Biodiversity, Pattern and Process in the European Hotspot* (Griffiths, H. I. & Kryštufek, B., eds), pp. 261–272. Dordrecht, Boston and London: Kluwer Academic Publishers.

Hadžišče, S. (1960). Zur Kenntnis der Gattung *Salmothymus* Berg. *Zavod za Ribarstvo na NR Makedonija-Skopje* **3,** 39–50.

Hadžišče, S. (1961). Zur Kenntnis des *Salmothymus ohridanus* (Steindachner) (Pisces, Salmonidae). *Internationale Vereinigung für theoretische und angewandte Limnologie Verhandlungen* **14,** 785–791.

Hoelzel, A. R., Hancock, J. M. & Dover, G. A. (1993). Generation of VNTRs and heteroplasmy by sequence turnover in the mitochondrial control region of two elephant seal species. *Journal of Molecular Evolution* **37,** 190–197.

Hoelzel, A. R., Lopez, J. V., Dover, G. A. & O'Brien, S. J. (1994). Rapid evolution of a heteroplasmic repetitive sequence in the mitochondrial DNA control region of carnivores. *Journal of Molecular Evolution* **39,** 191–199.

Kocher, T. D., Thomas, W. K., Meyer, A., Edwards, S. V., Paabo, S., Villablanca, F. X. & Wilson, A. C. (1989). Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences of the USA* **86,** 6196–6200.

Korniushin, A. V. (2004). The bivalve mollusc fauna of ancient lakes in the context of the historical biogeography of the Balkan region. In *Balkan Biodiversity, Pattern and Process in the European Hotspot* (Griffiths H. I. & Kryštufek, B., ed.), pp. 219–241. Dordrecht, Boston and London: Kluwer Academic Publishers.

Kottelat, M. (1997). European freshwater fishes; An heuristic checklist of the freshwater fishes of Europe (exclusive of former USSR), with an introduction for non-systematists and comments on nomenclature and conservation. *Biologia* **52** (Suppl. 5), 1–271.

Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. (2001). MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17,** 1244–1245.

Martens, K., Coulter, G. & Goddeeris, B. (1994). Speciation in Ancient Lakes-40 years after Brooks. *Archive für Hydrobiologie* **44,** 75–96.

Miller, S. A., Dykes, D. D. & Polesky, H. F. (1988). A simple salting out procedure from human nucleated cells. *Nucleic Acid Research* **16,** 1215.

Mundy, N. I. & Helbig, A. J. (2004). Origin and evolution of tandem repeats in the mitochondrial DNA control region of shrikes (*Lanius* spp.). *Journal of Molecular Evolution* **59,** 250–257.

O'Reilly, P. T., Hamilton, L. C., McConnell, S. K. & Wright, J. M. (1996). Rapid analysis of genetic variation in Atlantic salmon (*Salmo salar*) by PCR multiplexing of dinucleotide and tetranucleotide microsatellites. *Canadian Journal of Fisheries and Aquatic Sciences* **53,** 2292–2298.

Osinov, A. & Bernatchez L. (1996). Atlantic and Danubian phylogenetic groupings of brown trout (*Salmo trutta* L.) complex: genetic divergence, evolution, and conservation. *Journal of Ichthyology* **36,** 762–786.

Phillips, R. B., 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.
Phillips, R. B., Matsuoka, M. P., Konkol, N. R. & McKay, S. (2004). Molecular systematics and evolution of the growth hormone introns in the Salmoninae. *Environmental Biology of Fishes* **69,** 422–440.
Posada, D. & Crandall, K. A. (1998). Modeltest: Testing the model of DNA substitution. *Bioinformatics* **14,** 817–818.
Poteaux, C., Bonhomme, F. & Berrebi, P. (1999). Microsatellite polymorphism and genetic impact of restocking in Mediterranean brown trout (*Salmo trutta* L.). *Heredity* **82,** 645–653.
Pravdin, I. F. (1966). *Rukovodstvo Po Izucheniyu Rib*. Moscow: Pischevaya Promyshlennost Press. (in Russian).
Rakaj, N. & Flloko, A. (1995). Conservation status of freshwater fish of Albania. *Biological Conservation* **72,** 195–199.
Rand, D. M. & Harrison, R. G. (1989). Molecular population genetics of mtDNA size variation in crickets. *Genetics* **121,** 551–569.
Rexroad, C. E., Coleman, R. L., Hershberger, W. K. & Killefer, J. (2002). Rapid communication: Thirty-eight polymorphic microsatellite markers for mapping in rainbow trout. *Journal of Animal Science* **80,** 541–542.
Rice, W. R. (1989). Analysing tables of statistical tests. *Evolution* **43,** 223–225.
Roukonen, M. & Kvist, L. (2002). Structure and evolution of the avian mitochondrial control region. *Molecular Phylogenetics and Evolution* **23,** 422–432.
Rousset F. (1996). Equilibrium values of measures of population subdivision for stepwise mutation processes. *Genetics* **142,** 1357–1362.
Savolainen, P., Arvestad, L. & Lundeberg, J. (2000). MtDNA tandem repeats in domestic dogs and wolves: mutation mechanism studied by analysis of the sequence of imperfect repeats. *Molecular Biology and Evolution* **17,** 474–488.
Sell, J. & Spirkovski, Z. (2004). Mitochondrial DNA differentiation between two forms of trout *Salmo letnica*, endemic to the Balkan Lake Ohrid, reflects their reproductive isolation. *Molecular Ecology* **13,** 3633–3644.
Slettan, A., Olsaker, I. & Lie, O. (1995). Atlantic salmon, *Salmo salar*, microsatellites at the SSOSL25, SSOSL85, SSOSL311, SSOSL417 loci. *Animal Genetics* **26,** 281–282.
Slettan, A., Olsaker, I. & Lie, O. (1996). Polymorphic Atlantic salmon, *Salmo salar* L., microsatellites at the SSOSL438, SSOSL439 and SSOSL444 loci. *Animal Genetics* **27,** 57–58.
Snoj, A., Pohar, J. & Dovc, P. (1997). The first microsatellite DNA marker BFRO001 for Marble trout. *Journal of Animal Science* **75,** 1983.
Snoj, A., Jug, T., Melkič, E., Šušnik, 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.
Šorić, V. M. (1990). Ichthyogauna of the Ohrid-Drim-Skadar system. *Icthyologia* **22,** 31–43.
Stanković, S. (1960). *The Balkan Lake Ohrid and its Living World*. The Hague: W Junk.
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.
Weiss, S., Persat, H., Eppe, R., Schlotterer, C. & Uiblein, F. (2002). Complex patterns of colonization and refugia revealed for European grayling *Thymallus thymallus*, based on complete sequencing of the mitochondrial DNA control region. *Molecular Ecology* **11,** 1393–1407.

Electronic References

Gharbi, K. (2001). Construction d'une carte génétique partielle du génome tétraploide de la truite commune (*Salmo trutta*). PhD thesis. Available from http://www.inra.fr/theses/these-integrale/Theses/gharbi/html/these_front.html

Goudet, J. (2001). FSTAT, Version 2.9.3: a Program to Estimate and Test Gene Diversities and Fixation Indices. Available from http://www.unil.ch/izea/softwares/fstat.html