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RESEARCH PAPER

Lack of reproductive isolation between the Western and Eastern phylogroups of the tench

Zdeněk Lajbner · Klaus Kohlmann · Otomar Linhart · Petr Kotlík

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Abstract The Eurasian range of the tench distribution is subdivided into deeply divergent Western and Eastern phylogroups evidenced by nuclear and mitochondrial DNA sequence markers. A broad zone of overlap exists in central and western Europe, suggesting post-glacial contact with limited hybridisation. We conducted a population genetic test of this indication that the two phylogroups may represent distinct species. We analysed variation at introns of nuclear genes, microsatellites, allozymes and mitochondrial DNA in populations from two postglacial lakes within the contact zone in Germany. The test is based on the expectation that in the presence of strong barriers to reproduction, a hybrid population will show genome-wide associations among alleles and genotypes from each phylogroup even after hundreds of generations of interbreeding. In contrast to this expectation, no consistent significant deviations from linkage and Hardy–Weinberg equilibria were found. Samples from both lakes did show significant disequilibria but they were limited to individual loci and were not concordant between populations, and were not robust to the method used. The single consistent association can be attributed to physical linkage between two microsatellite loci. Thus, results of our study support the hypothesis of free interbreeding between the two phylogroups of tench. Therefore, although the phylogroups may be considered as separate phylogenetic species, the present data suggest that they are a single species under the biological species concept.

Keywords Allozymes · Microsatellites · mtDNA · Disequilibrium · Hybridization · *Tinca tinca*

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Introduction

Most freshwater fish species in Eurasia show phylogeographic subdivisions of their geographic ranges that developed in response to recurrent isolation in glacial

refugia during the Pleistocene (Hewitt 2004). These range shifts and the accompanying demographic changes resulted in divergence between refugial populations, which in some species may have proceeded towards speciation (see Bernatchez and Wilson 1998; Knowles 2001; Carstens and Knowles 2007). A recent phylogeographic study of the tench *Tinca tinca* (L.) demonstrated that the Eurasian range of this species is subdivided into deeply divergent Western and Eastern phylogroups (i.e. clades with geographically adjacent distributions), evidenced by phylogenetic analysis of sequence variation at introns of nuclear genes (actin, S7 ribosomal protein and ATP synthase beta subunit) and at a mitochondrial (mt) DNA gene (cytochrome *b*) (Lajbner et al. 2007). Similar to other European freshwater fish species (Durand et al. 1999; Nesbø et al. 1999; Kotlík and Berrebi 2001), these phylogroups probably diverged after a colonization of western Europe from the Black Sea basin during a Pleistocene interglacial and their subsequent separation by cold periods, conforming to the 'chub' paradigm pattern (Hewitt 2004). A broad zone of overlap was formed upon the geographic contact between the tench phylogroups in central and western Europe following their postglacial expansion from two principal freshwater refugia, the Ponto-Caspian refugium (Bănărescu 1991; Kotlík et al. 2004) and the western European refugium (e.g. Durand et al. 1999; Nesbø et al. 1999; Kotlík and Berrebi 2001). The sequence divergence between the tench phylogroups at mtDNA (1.3% for cytochrome *b* gene) approaches divergence among distinct fish species (Hendry et al. 2000a) and it roughly corresponds to a separation time for at least 750,000 years (see Waters et al. 2007). Allopatric speciation takes usually a prolonged time (McCune and Lovejoy 1998) but there are exceptions (Near and Benard 2004), and in sympatry mating barriers can evolve in just few generations (Hendry et al. 2000b). The existence of broad contact zone with the presence of individuals of apparently hybrid ancestry (i.e. heterozygous for alternate phylogroup-specific alleles at nuclear loci or homozygous at a nuclear locus but with mtDNA of the opposite phylogroup) suggests that there is incomplete reproductive isolation between the tench phylogroups. On the other hand, the fact they have remained effectively allopatric outside the contact zone suggests the existence of mechanisms restricting introgression.

The present study examines the existence of barriers to reproduction between the Western and Eastern phylogroups of tench in two lakes within the zone of their postglacial contact. The Grosser Felchowsee and Kleiner Döllnsee lakes are situated in north-eastern Germany in an area covered by the Scandinavian ice sheet during the Weichselian glaciation (Ehlers et al. 2004). The phylogeographic study revealed the presence of markers characteristic of the Western as well as the Eastern phylogroup in both lakes (Lajbner et al. unpublished). The lakes could only be colonised by tench after the deglaciation, and the populations inhabiting the lakes today most likely were not founded before the end of the Younger Dryas (Jahns 2000), about 11,500 years ago (Muscheler et al. 2008). Nevertheless, the period since then corresponds to roughly 3,000 generations of tench, and the populations founded by a mixture of the Western and Eastern phylogroups should not show genome-wide associations among alleles and genotypes from each phylogroup if they have been freely interbreeding (Nagylaki 1976, 1977; Barton and Gale 1993). If, on the contrary, the phylogroups show genetic incompatibilities in terms of differential frequency or viability of homospecific versus heterospecific crosses, the populations should display genome-wide linkage and Hardy–Weinberg disequilibria, even after the many generations of interbreeding (e.g. Lajbner et al. 2009). To rectify this issue, tench sampled from these lakes were genotyped for two introns of nuclear genes (actin and S7 ribosomal protein) and for a mtDNA. These three markers are fully diagnostic between the two phylogroups in that they posses alternate, DNA sequence-based classes of alleles (Lajbner et al. 2007), which were distinguished here by restriction fragment length polymorphism (RFLP) assay. To provide a broader genomic coverage of markers, these data were analysed along with genotypes for the same individuals at additional fourteen genetic loci with unknown phylogroup specificity (allozyme and microsatellite), which were scored by starch electrophoresis (allozymes for the Felchowsee individuals) or were available from earlier studies (Kohlmann and Kersten 1998, 2006; Kohlmann et al. 2007, 2009).

The principal question addressed by this study is whether there is evidence of two genetically distinct reproductive units in each lake. More specifically, the results are used to determine whether the lakes show

signs of a hybrid population structure (i.e. purebred Western, purebred Eastern, F_1 hybrids etc.), and if there are consistent (between lakes and across loci and methods) deviations from linkage and Hardy–Weinberg equilibria in these lakes that could be attributed to the existence of barriers to merging of gene pools of the two founding phylogroups.

Materials and methods

Data acquisition

The study was conducted on 49 tench collected from a wild population inhabiting Lake Grosser Felchowsee (160 ha; 53°3′N, 14°8′E). This sampling was supplemented by an additional 19 individuals from Lake Kleiner Döllnsee (25 ha; 52°59′N, 13°34′E). Both lakes are situated in Germany in the north-eastern part of the Oder River drainage of the Baltic Sea basin, in the lowland area rich in lakes and wetlands. There have probably never been introductions of foreign tench into the lakes or supportive artificial reproduction of the indigenous tench from the lakes (T. Löwe, Lake Felchowsee owner, personal communication).

Recently conducted large scale phylogeographic study (Lajbner et al. 2007) gives a possibility to discriminate the two evolutionary lineages of tench on the basis of two nuclear markers and one mitochondrial marker, which can be easily scored by RFLP. A restriction map was generated for each marker using the CLC Free Workbench 4.5.1 (CLC bio) from haplotype sequences of *T. tinca* (Lajbner et al. unpublished) and endonucleases digesting the markers at lineage specific positions were selected. The phylogroup specific restriction endonuclease Eco52I was selected for the 2nd intron of the actin gene and MboII for the 1st intron of the S7 ribosomal protein (RPS7) gene. Both endonucleases were predicted to yield phylogroup-specific RFLP profiles following digestion of the respective polymerase chain reaction (PCR) products due to restriction sites present only in one of the phylogroups. The restriction endonuclease AluI was predicted to yield phylogroup-specific RFLP profiles following digestion of a PCR product of the mtDNA cytochrome *b* gene due to a diagnostic restriction site, which unambiguously identified each individual to either Eastern or Western phylogroup maternal ancestry.

Total DNA of fish from Felchowsee and Döllnsee was extracted from ethanol-preserved muscle tissue or from fin clips by using Dneasy Tissue Kit (Qiagen). A part of nuclear DNA containing 2nd intron of actin gene (336 bp) was amplified using primers Act-2-R and Act-2-F designed by Touriya et al. (2003) and another part containing 1st intron of RPS7 gene (900 total bp) using primers S7RPEX2R and S7RPEX1F (Chow and Hazama 1998). In addition, an approximately 1,225 bp long portion of mtDNA containing entire gene for cytochrome *b* was amplified for fish from Felchowsee using primers GluF and ThrR (Machordom and Doadrio 2001). For the sake of simplicity, the PCR program was unified for all markers and contained 5 min of initial denaturation at 95°C, touch-down profile of 1 min at 94°C, two cycles at 60–56°C (2°C/cycle) for 1 min 30 s, and 2 min at 72°C followed by 30 cycles with annealing temperature held at 54°C. The PCR reaction mix consisted of 12.5 mm^3 of Top-Bio PPP Master Mix (Top-Bio, Prague, Czech Republic), 10 pmol of each primer, 0.2 μg of DNA and demineralised water up to 25 mm^3. For RFLP analysis, 4 mm^3 of the PCR products were digested for 10 h at 37°C in 10 mm^3 volumes containing 4.7 mm^3 of demineralised water and 1 mm^3 of Y^+/Tango buffer with 0.3 mm^3 of the restriction endonucleases AluI, Eco52I or MboII (Fermentas, Vilnius, Lithuania) for fragments in the same order as listed above and than deactivated at 65°C for 20 min. Restriction fragments were separated on 2% agarose gel containing 2 mm^3 of GoldView (SBS Genetech, Shanghai, China).

Samples from Felchowsee were analysed for their genetic variability in 11 enzymatic systems representing 24 gene loci by horizontal starch gel electrophoresis (Aebersold et al. 1987) (Table 1). Staining of all but two enzymes followed the standard procedures of Shaw and Prasad (1970) and the modified protocol of Vuorinen (1984). Creatine kinase was visualised using general protein staining (amido black). Superoxide dismutase appeared as light, whitish spots on gels stained for alcohol dehydrogenase, glycerol-3-phosphate dehydrogenase or phosphoglucomutase, respectively. Enzyme banding patterns were read by using the tench gene nomenclature of Šlechtová et al. (1995), which follows the rules suggested by Shaklee et al. (1990). Alleles were named according to their relative electrophoretic mobilities.

Table 1 Enzymes and tissues examined, number of loci screened and buffer systems used

Enzyme	E.C. number	Abbreviation	Tissue	Number of loci	Buffer[a]
Aspartate aminotransferase	2.6.1.1.	mAAT	Muscle	2	B
		sAAT	Muscle/liver	1	B
Alcohol dehydrogenase	1.1.1.1.	ADH	Liver	1	C
Creatine kinase	2.7.3.2.	CK	Muscle	1	A
Glycerol-3-phosphate dehydrogenase	1.1.1.8.	G3PDH	Muscle/liver	2	C
Glucose-6-phosphate isomerase	5.3.1.9.	GPI	Muscle/liver	2	A
Isocitrate dehydrogenase	1.1.1.42.	mIDHP	Muscle	2	C
		sIDHP	Liver	2	B
Lactate dehydrogenase	1.1.1.27.	LDH	Muscle/liver	3	B
Malate dehydrogenase	1.1.1.37.	mMDH	Muscle/liver	2	B
		sMDH	Muscle/liver	2	B
Phosphogluconate dehydrogenase	1.1.1.44.	PGDH	Liver	1	B + C
Phosphoglucomutase	5.4.2.2.	PGM	Muscle/liver	2	C
Superoxide dismutase	1.15.1.1.	SOD	Liver	1	C

[a] Buffers: *A* Tris–citric acid, pH 8.5 (gel) and lithium hydroxide—boric acid, pH 8.1 (tray; Ridgway et al. 1970), *B* Citric acid—morpholine, pH 6.5 (Clayton and Tretiak 1972, modified by Vuorinen 1984), *C* Tris–citric acid, pH 7.1 (Shaw and Prasad 1970)

Additional raw data for the same allozyme loci for fish from Döllnsee and 6 microsatellite loci for fish from both lakes were taken from studies of Kohlmann and Kersten (1998, 2006) and Kohlmann et al. (2007, 2009). Microsatellite locus MTT8 was inferred to contain a null allele (Kohlmann et al. 2009) and was therefore discarded from most analyses. Allelic frequencies of MTT6 and MTT2 were compared with raw data of Kohlmann et al. (2009) from Las Vegas del Guadiana fish farm in Spain (Bada), Wuhan in China (Chin) and Lake Sapanca in Turkey (Turk) that appeared to contain only Eastern phylogroup alleles (Lajbner et al. unpublished).

Data analyses

For each population, variation at polymorphic loci was summarized as Nei's unbiased expected heterozygosity or gene diversity (Nei 1987).

Three types of exact test of Hardy–Weinberg equilibrium were conducted for each population by using Genepop 4.0 (Rousset 2008), which all assume the same null hypothesis (random union of gametes) but differ in the construction of the rejection zone. In the exact probability test (e.g. Haldane 1954; Weir 1996), the probability of the observed sample is used to define the rejection zone, and the *P*-value of the test corresponds to the sum of the probabilities of all tables (with the same allelic counts) with the same or lower probability. Two variants of the more powerful score test (*U* test) were run, which assumed, respectively, heterozygote excess or heterozygote deficiency as the alternative hypothesis to panmixia (Rousset and Raymond 1995). The Markov chain algorithm to estimate without bias the exact *P*-value of this test (Guo and Thompson 1992) was conducted by 1,000 batches of 20,000 iterations following 20,000 dememorization steps.

Wright's *F*-statistics was used as another means of quantifying the conformity of genotype frequencies to Hardy–Weinberg proportions and to test the existence of geographical subdivision of populations. Two parameters were estimated for the polymorphic loci according to Weir and Cockerham (1984) with the Genetix software package, v.4.05 (Belkhir et al. 2004). The inbreeding coefficient F_{IS} was estimated by the estimator f, and the fixation index F_{ST} by the estimator θ (Weir and Cockerham 1984). The significance of the multilocus estimates was assessed by a permutation test using a 20,000 randomised data set generated by permuting the alleles among individuals for F_{IS} and the individuals among the samples for F_{ST} (Dallas et al. 1995; Balloux and Lugon-Moulin 2002).

Exact multilocus tests for association between alleles were also computed using software MLD (Zaykin et al. 1995) allowing haplo-diploid data

combination. In this test, the proportion of 20,000 permuted multilocus genotypic arrays as probable or less probable than the sample forms an estimate of the significance level. Pairwise genotypic and allelic linkage disequilibria were calculated by Black and Krafsur (1985) method of Linkdos (Garnier-Géré and Dillmann 1992) implemented in Genetix 4.05 (Belkhir et al. 2004), and using Cockerham and Weir's (1977) coefficient of gametic disequilibrium for each pair of loci (D_{ij}) and Weir's (1979) correlation coefficient between alleles at two loci (R_{ij}). The significance of the genotypic linkage disequilibria was calculated using 10,000 permutations of genotypes among individuals within each population while significance of allelic associations were estimated by the chi-square test (Weir 1979). Pairwise genotypic associations among all loci (nuclear-encoded and mitochondrial) were also calculated by Linkdos (Garnier-Géré and Dillmann 1992) as implemented in Genepop 4.0 (Rousset 2008). Contingency tables were created for all pairs of loci for each lake and a G test was computed for each table using the Markov chain algorithm of Raymond and Rousset (1995).

Bayesian method and the program NewHybrids v.1.1 (Anderson and Thompson 2002) were used for quantifying the level of certainty that each individual belongs to each of pre-specified genotype classes. The method does not require that allele frequencies of each of the species are known and the loci used do not necessarily need to be diagnostic. Rather, it uses a Markov chain Monte Carlo simulation to integrate over possible values of the model parameters (i.e. the proportion of individuals from the different genotype classes and the allele frequencies of each species), and estimates the posterior probability that an individual belongs to each genotype class (Anderson and Thompson 2002). The inheritance model implemented assumes that a sample has been drawn from a mixed population of two species with unknown proportions of individuals from the different hybrid classes. Although it is generally possible to consider as many hybrid classes as needed, the finite number of available loci is not sufficient to reliably distinguish between these numerous categories, and it is thus more appropriate to concentrate on the early generation hybrid classes (Boecklen and Howard 1997; Epifanio and Philipp 1997; Rieseberg and Linder 1999). Therefore, only those genotype classes that could occur after up to three generations of crossing between the parental species were allowed (pure Western phylogroup, pure Eastern phylogroup, F_1 hybrid, F_2 hybrid, BC_1 to Western phylogroup, BC_1 to Eastern phylogroup and their crosses). Three separate NewHybrids analyses were run, the first for the Felchowsee and Döllnsee together, the second for the Felchowsee alone, and the third for the Döllnsee alone. For all analyses the Markov chain was run with a burn-in period of 100,000 iterations and 1,000,000 iterations following the burn-in, and assuming uninformative Jeffreys-type priors on the parameters. Each analysis was run several times to assess convergence. The analysis was considered to have converged upon a stationary distribution if the independent runs generated similar results.

We determined the number of subpopulations of tench within each lake using a statistical procedure (Pritchard et al. 2000) that attempts to minimize disequilibrium (Hardy–Weinberg and linkage) within groupings. The number of subpopulations (K) with the highest posterior probability was estimated by using the program Structure 2.2 (Pritchard et al. 2000; Falush et al. 2003, 2007). The admixture model and the option of correlated allele frequencies between populations (also called the F-model) were selected because it is considered the superior model for detecting structure even among closely related populations (Falush et al. 2003). Markov Chain Monte Carlo runs consisted of 100,000 burn-in iterations followed by 1,000,000 iterations. We explored K in the range from one to nine and performed 10 runs for each K value in each lake separately.

Results

Hardy–Weinberg and linkage equilibrium

Tench populations from Felchowsee and Döllnsee were moderately but significantly differentiated ($F_{ST} = 0.012$, $P < 0.05$). Samples from each lake were therefore treated separately in the analyses.

Heterozygote deficiencies measured by the inbreeding coefficient and by the exact tests of Hardy–Weinberg equilibrium (probability and scores test) revealed a significant deviation of genotype frequencies from Hardy–Weinberg proportions in both lakes. However, the loci that showed deviations in Felchowsee were not the same as the loci that

showed significant patterns in Döllnsee (Table 2). Multilocus heterozygote deficiencies measured by the inbreeding coefficient revealed a significant deviation from Hardy–Weinberg proportions in Felchowsee ($F_{IS} = 0.079$, $P < 0.01$) but not in Döllnsee ($F_{IS} = 0.040$, $P > 0.05$). The exclusion of locus MTT8 from this analysis slightly reduced the values of the inbreeding coefficient but did not reduce the levels of significance of these findings in Felchowsee ($F_{IS} = 0.059$, $P < 0.05$) or in Döllnsee ($F_{IS} = 0.032$, $P > 0.05$). The significant indication of multilocus heterozygote deficiency is further suported in Felchowsee ($P < 0.001$) but not in Döllnsee ($P > 0.05$) by the multilocus score test under the heterozygote deficiency alternative hypothesis. The result remained significant after the exclusion of MTT8 locus in Felchowsee ($P < 0.05$) and insignificant in Döllnsee ($P > 0.05$).

Multilocus test of linkage disequilibria detected significant associations in both lakes (MLD exact test, $P < 0.05$). However, few significant pairwise genotypic disequilibria were found (Table 3) and no significant cytonuclear disequilibria were detected. Only the disequilibrium between microsatellite loci MTT2 and MTT6 was consistently significant in both lakes, independent on the method used (Table 3). The associated alleles of these loci were the same in both lakes (Table 4), and they were indistinguishable in size from alleles fixed in three putatively pure Eastern populations (Bada, Chin and Turk) analysed by Kohlmann et al. (2009).

Population and hybrid structure

Partitioning of the tench populations in two distinct reproductive units (i.e. subpopulations) was not supported by the Structure analysis. Although the prior parameters for the F model (gamma distribution with mean 0.01 and SD 0.05) were chosen to allow the existence of two populations even with very similar allele frequencies, in both populations the highest log likelihood value (Felchowsee: mean −1,168.467, SD 0.327; Döllnsee: mean −406.750, SD 0.565) was found for $K = 1$. Log likelihood values for the population consisting of two (Felchowsee: mean −1,175.483, SD 4.080; Döllnsee: mean −407.420, SD 0.258) or three separate reproductive units (Felchowsee: mean −1,190.433, SD 9.279; Döllnsee: mean −408.840, SD 0.559) were lower than for $K = 1$, and higher values of K received progressively decreasing probabilities.

The NewHybrids analysis did not detect any individual that could be classified as either purebred Western or purebred Eastern or any of the early-generation hybrids. Instead, the analyses assigned all individuals from both lakes as advanced backcrosses to Western phylogroup. Genotypically, this category corresponds to the product of mating of the first-generation backcrosses of a backcross-to-Western type among themselves. The posterior probability of assignment to this category was similar for individuals from Felchowsee (mean 0.884, SD 0.066) and Döllnsee (mean 0.735, SD 0.021) and for a pooled sample (0.923, SD 0.039).

Discussion

Despite their high evolutionary divergence (1.3% for cytochrome *b* gene) that compares with genetic distance between species (Avise et al. 1998), two phylogroups of tench emanating from different Pleistocene refugia form a broad contact zone in Europe composed of individuals of mixed ancestry (Lajbner et al. unpublished). The present study found evidence that these phylogroups are not separated by strong barriers to reproduction and that they merged back into a single population after colonization of the same postglacial lake.

If the phylogroups evolved genetic incompatibilities during their refugial isolation, the current hybrid populations should consistently display significant associations among alleles and genotypes from each phylogroup caused by lowered frequency and/or viability of crosses between the phylogroups (e.g. Caputi et al. 2007; Städler et al. 2008; Lajbner et al. 2009). If, on the contrary, they freely interbreed, current populations that were postglacially founded by both phylogroups should not show genome-wide associations anymore as the result of inter-locus recombination over the many generations of interbreeding (see Templeton 2006).

Results of the various analyses in this study strongly supported the hypothesis of free interbreeding between the phylogroups as opposed to strong barriers to reproduction. The Structure analysis (Pritchard et al. 2000) suggested that tench in each lake corresponded with the highest probability to a

Table 2 Variation at each polymorphic locus and conformity to Hardy–Weinberg expectations evaluated with four different tests

Marker	Felchowsee								Döllnsee							
	Sample size	Number of alleles	Gene diversity	F_{IS}	F_{IS} (P)	HW (P)	HE (P)	HD (P)	Sample size	Number of alleles	Gene diversity	F_{IS}	F_{IS} (P)	HW (P)	HE (P)	HD (P)
Actin	36	2	0.3658	−0.2963	0.0871	0.1555	0.0838	1.0000	17	2	0.4011	−0.0275	0.7109	1.0000	0.7120	0.7631
RPS7	36	2	0.3658	0.3196	0.0714	0.0724	0.9901	0.0727	17	2	0.2139	−0.1065	0.8229	1.0000	0.8212	1.0000
MTT1	47	7	0.7884	−0.1078	0.0980	0.4743	0.0914	0.9154	19	5	0.7397	0.1496	0.1975	**0.0415**	0.9607	**0.0396**
MTT2	49	2	0.3737	0.1273	0.2991	0.4415	0.8999	0.2947	19	2	0.4623	−0.2611	0.2706	0.3449	0.2683	0.9586
MTT5	49	4	0.6449	0.1149	0.1397	0.2172	0.8243	0.1739	19	4	0.6615	0.2895	**0.0456**	0.0890	0.9806	**0.0267**
MTT6	49	5	0.7084	0.1950	**0.0236**	0.2101	0.9887	**0.0108**	18	6	0.6238	−0.1632	0.2273	0.3824	0.2552	0.8516
MTT8	49	3	0.2401	0.5775	**0.0001**	**0.0002**	0.9999	**0.0002**	17	3	0.1693	0.3118	0.0898	0.0909	0.9690	0.0931
MTT9	49	19	0.9007	0.0488	0.1945	0.8056	0.8431	0.1111	18	9	0.8587	−0.0362	0.5197	0.5184	0.4565	0.5859
*sAAT**	49	2	0.1851	0.1193	0.3969	0.3992	0.9390	0.3978	19	2	0.3087	−0.2000	0.5133	1.0000	0.5105	1.0000
*ADH**	49	2	0.2897	0.0850	0.4307	0.6160	0.8669	0.4287	19	2	0.2347	−0.1250	0.7404	1.0000	0.7416	1.0000
*GPI-1**	49	2	0.0978	0.3766	0.1044	0.1015	0.9984	0.1016	19	1	0					
*GPI-2**	48	2	0.2211	0.0581	0.5443	0.5432	0.8642	0.5449	19	2	0.3087	0.4953	0.0796	0.0762	0.9976	0.0763
*mIDHP-2**	49	2	0.2619	0.1442	0.2922	0.2936	0.9365	0.2929	19	2	0.2347	0.3333	0.2618	0.2587	0.9887	0.2585
*LDH-2**	49	2	0.0978	−0.0435	0.8994	1.0000	0.8982	1.0000	19	1	0					
*PGDH**	49	2	0.3158	0.0310	0.5692	1.0000	0.7611	0.5713	19	2	0.3087	−0.2000	0.5187	1.0000	0.5103	1.0000
*SOD**	49	2	0.3518	−0.0447	0.5504	1.0000	0.5588	0.7601	19	2	0.3983	0.2117	0.3537	0.5498	0.9362	0.3492

Only eight out of 24 examined allozyme loci were polymorphic in Felchowsee and six in Döllnsee. Test probabilities (P) are given for the inbreeding coefficient F_{IS}, for the exact probability test of Hardy–Weinberg proportions (HW), and for two variants of the score test assuming respectively heterozygote excess (HE) or heterozygote deficiency (HD) as the alternative hypothesis to panmixia. Probability values lower than 5% are in bold

Locus symbols are italicized for allozyme loci and include an asterisk to distinguish them from abbreviations of the enzymes they code (see Shaklee et al. 1990)

Table 3 Linkage disequilibria in Döllnsee (above the diagonal) and Felchowsee (below the diagonal) expressed as average correlation coefficients between alleles (R_{ij}) at each pair of loci

Marker	Actin	RPS7	MTT1	MTT2	MTT5	MTT6	MTT8	MTT9	*sAAT**	*ADH**	*GPI-1**	*GPI-2**	*mIDHP-2**	*LDH-2**	*PGDH**	SOD*
Actin	–	0.0286	0.1922	0.1669	0.2772	0.3233	0.3178	0.1845	0.1480	0.3064	–	0.0639	0.4006	–	0.0617	0.4041
RPS7	0.0857	–	0.3006	0.5243	0.2820	0.3031	0.1839	0.2956	0.4931	0.3804	–	0.4711	0.0159	–	0.1057	0.2885
MTT1	0.2040	0.1240	–	0.2091	0.1614	0.2093	0.1466	0.2045	0.3242	0.2660	–	0.1900	0.2066	–	0.2714	0.2460
MTT2	0.0863	0.1308	0.1871	–	0.1410	**0.4322**$^{**/**}$	0.0898	0.1551	0.3636	0.3514	–	0.2634	0.0754	–	0.3636	0.1674
MTT5	0.1260	0.2043	0.0927	0.1602	–	0.1732	0.2999	**0.2889**$^{*/NS}$	0.1944	0.2250	–	0.1368	0.1617	–	0.1997	0.2304
MTT6	0.1319	0.2000	**0.1452**$^{NS/*}$	**0.3166**$^{***/***}$	0.1187	–	0.1041	0.2699	0.2556	0.1894	–	**0.4008**$^{*/NS}$	0.2053	–	0.1707	0.1783
MTT8	**0.2775**$^{*/NS}$	**0.2216**$^{NS/**}$	0.1703	0.0552	0.1376	0.1456	–	0.1984	0.1175	0.2196	–	0.2027	0.1574	–	0.2513	0.2589
MTT9	0.1663	0.1108	0.1139	0.1204	0.1256	0.1020	0.1070	–	0.2033	0.2796	–	0.2158	0.2781	–	0.1675	0.1638
*sAAT**	0.0266	0.0194	0.1176	0.2083	0.0546	0.1422	0.1917	0.1027	–	0.0413	–	0.1002	0.1773	–	0.1005	0.2235
*ADH**	0.0087	0.1460	0.1321	0.1358	0.1756	0.0670	0.1179	0.1354	0.0391	–	–	0.3491	0.3035	–	0.2203	0.4407
*GPI-1**	0.0162	0.1177	0.1336	0.0400	0.0528	0.0747	0.0988	0.1255	0.0026	0.1305	–	–	–	–	–	–
*GPI-2**	–	–	0.1189	0.1377	0.1650	0.0931	0.0925	0.1318	0.0489	0.1790	0.1512	–	0.0241	–	**0.0728**$^{NS/*}$	0.2073
*mIDHP-2**	0.0314	0.1416	0.1350	0.1403	0.1111	0.1189	0.0482	0.1280	0.0052	0.0141	0.0496	0.1017	–	–	0.0333	0.0552
*LDH-2**	0.1619	0.2501	0.1828	0.2622	0.0587	0.1419	0.1188	0.0999	0.0031	0.0911	0.0965	0.1608	0.1865	–	–	–
*PGDH**	0.0149	0.1447	0.1835	0.1323	0.1610	0.0672	0.1196	0.1427	0.0100	0.1058	0.1076	0.1183	0.0555	0.1293	–	0.0536
*SOD**	0.2381	0.1039	0.1137	0.0438	0.2070	0.0944	0.0115	0.1036	0.0411	0.0414	0.0754	0.1158	0.2187	**0.0294**$^{NS/*}$	0.1627	–

Superscript asterisks denote significance with the results obtained with the permutation method (Garnier-Géré and Dillmann 1992) separated by a slash mark (/) from the results obtained with the Markov chain method (Raymond and Rousset 1995) where at least one method gave a significant result (in bold). The values for the linkage disequilibrium measures between *GPI-2** and the two intron loci are missing due to missing data

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, non-significant

Table 4 Allelic associations between alleles at the microsatellite loci MTT2 and MTT6 expressed as correlation coefficients (R_{ij})

Alleles	Felchowsee		Döllnsee	
	R_{ij}	P	R_{ij}	P
236-160	0.6672	**0.0001**	0.7379	**0.0017**
236-164	–	–	0.2161	0.3592
236-168	–	–	0.1483	0.5293
236-170	−0.1424	0.3188	−0.4323	0.0667
236-172	−0.4675	**0.0011**	−0.5994	**0.0110**
236-174	−0.1123	0.4319	−0.2965	0.2084
236-176	−0.0707	0.6208	–	–
240-*160*	−0.6672	**0.0001**	−0.7379	**0.0017**
240-*164*	–	–	−0.2161	0.3592
240-168	–	–	−0.1483	0.5293
240-170	0.1424	0.3188	0.4323	0.0667
240-172	0.4675	**0.0011**	0.5994	**0.0110**
240-174	0.1123	0.4319	0.2965	0.2084
240-176	0.0707	0.6208	–	–

Significant probability values (at the 0.05 level) are in bold and alleles present in the three presumably pure Eastern populations (Bada, Chin and Turk) are in italics

single population at equilibrium (Hardy–Weinberg and linkage). The NewHybrids analysis (Anderson and Thompson 2002) did not detect any individual that could be classified as either purebred Western or purebred Eastern or any of the early-generation hybrids, and it assigned all individuals from both lakes as advanced backcrosses to the Western phylogroup. This suggests that the data does not fit well the NewHybrids model assuming two hybridizing populations, which is consistent with the result of the Structure analysis.

The conformity of genotype frequencies to Hardy–Weinberg proportions could not be rejected for the majority of loci by any of the methods. Although the samples from both lakes showed significant disequilibria at some loci, the patterns were not concordant: the loci that showed deviations from the Hardy–Weinberg or linkage equilibrium in Felchowsee were not the same as the loci that showed a significant disequilibrium in Döllnsee. Furthermore, whether a locus showed a significant deviation was dependent on the statistics used to quantify the conformity of the samples to the Hardy–Weinberg proportions (F_{IS} vs. exact test) or on the method used to estimate the significance of linkage disequilibria (permutations vs. Markov chain Monte Carlo approximation). Kohlmann et al. (2009) explained the heterozygote deficiency found in Felchowsee at the microsatellite locus MTT8 by the presence of a 'null' allele not amplified with the current primers, resulting in a biased estimate of population genotype frequencies at this locus.

Of all pairwise comparisons, only the microsatellite loci MTT2 and MTT6 were in significant linkage disequilibrium in both lakes and this pattern was not dependent on the method. Because no other pair of loci showed consistent disequilibria, it seems most reasonable to attribute the observed association between MTT2 and MTT6 to strong physical linkage of these loci (Page and Holmes 1998). The relative location of the markers in the tench genome is, however, currently unknown and this hypothesis needs further testing. Furthermore, the comparison with purebred Eastern populations suggest positive association of alleles at these loci within phylogroups.

Although the tench phylogroups formed a broad contact zone during their postglacial dispersions, they have remained effectively allopatric outside the contact zone. This is especially evident in the Eastern phylogroup that shows no signs of introgression with genes from the Western phylogroup throughout its broad distribution between the Danube River in the west to Lake Baikal in the east (Lajbner et al. unpublished). This is remarkable given that the present study demonstrated that the phylogroups are not reproductively isolated and their gene pools have effectively merged in mixed populations. Furthermore, it is reasonable to expect that similar patterns to that observed today were created by dispersion from the refugia also following earlier glacial maxima because the mtDNA divergence between the phylogroups covers multiple glacial-interglacial cycles (Hewitt 2004). What could have caused the refugial populations to retain their genetic integrity in face of recurrent contacts and interbreeding? The answer may be found in the dynamics of species response to changing climate. If, as appears to be the case, most species responded to glacier advances by extinction of populations in northerly areas with only populations in the vicinity or refugia surviving (Hewitt 2004), the admixed populations outside the refugia would have been extirpated at the onset of each glaciation, protecting the genetic purity of refugia (Hofreiter et al. 2004).

The tench phylogroups can be considered separate species according to the phylogenetic species concept (Mishler and Theriot 2000; Wheeler and Platnick 2000). Some genetic disequilibria were detected in natural hybrid populations but the pattern is not consistent between populations and across methods as would be expected in case of a strong reproductive barrier. Thus, the present data suggest that the tench can be considered a single species under the biological species concept (Mayr 1942, 1963).

The fact that tench phylogroups can interbreed but remained distinct in the refugia has important practical implications. For example, populations in the vicinity of the refugia may show physiological adaptations to different selective environments that could be lost if these populations were extirpated (e.g. by anthropogenic habitat change) or admixed by stocking with individuals of the opposite phylogroup or the mixed ancestry (Allendorf et al. 2001). On the other hand, since the two phylogroups readily form mixed populations without detectable negative fitness consequences and tench are widely used for aquaculture, controlled genetic experiments can be envisaged to identify the genes underlying important physiological or structural phenotypes (Nikinmaa and Waser 2007). Furthermore, many tench populations in Europe are admixed, which offers a unique opportunity to identify the genetic basis of phenotypic traits by the admixture mapping approach (Buerkle and Lexer 2008). Tench phylogroups, therefore, represent unique genetic resources and a valuable new model for applied genetic research.

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References

Aebersold PB, Winans GA, Teel DJ, Millner GB, Utter FM (1987) Manual for starch gel electrophoresis: A method for the detection of genetic variation. NOAA Technical Report NMFS 61. U.S. Department of Commerce

Allendorf FW, Leary RF, Spruell P, Wenburg JK (2001) The problems with hybrids: setting conservation guidelines. Trends Ecol Evol 16:613–622

Anderson EC, Thompson EA (2002) A model-based method for identifying species hybrids using multilocus genetic data. Genetics 160:1217–1299

Avise JC, Walker D, Johns GC (1998) Speciation durations and Pleistocene effects on vertebrate phylogeography. Proc R Soc Lond B 265:1707–1712

Balloux F, Lugon-Moulin N (2002) The estimation of population differentiation with microsatellite markers. Mol Ecol 11:155–165

Bănărescu PM (1991) Zoogeography of fresh waters, 2: distribution and dispersal of fresh water animals in North America and Eurasia. AULA-Verlag, Wiesbaden

Barton NH, Gale KS (1993) Genetic analysis of hybrid zones. In: Harrison RG (ed) Hybrid zones and the evolutionary process. Oxford University Press, Oxford, pp 13–45

Belkhir K, Borsa P, Chikhi L, Raufaste N, Bonhomme F (2004) Genetix 4.05, Logiciel sous Windows pour la Génétique des Populations. Laboratoire Genome, Populations, Interactions, CNRS UMR. 5171. Université de Montpellier II, Montpellier

Bernatchez L, Wilson CC (1998) Comparative phylogeography of nearctic and palearctic freshwater fishes. Mol Ecol 7:431–452

Black WC IV, Krafsur ES (1985) A FORTRAN program for the calculation and analysis of two-locus linkage disequilibrium coefficients. Theor Appl Genet 70:491–496

Boecklen WJ, Howard DJ (1997) Genetic analysis of hybrid zones: numbers of markers and power of resolution. Ecology 78:2611–2616

Buerkle CA, Lexer C (2008) Admixture as the basis for genetic mapping. Trends Ecol Evol 23:686–694

Caputi L, Andreakis N, Mastrototaro F, Cirino P, Vassillo M, Sordino P (2007) Cryptic speciation in a model invertebrate chordate. Proc Natl Acad Sci USA 104:9364–9369

Carstens BC, Knowles LL (2007) Shifting distributions and speciation: species divergence during rapid climate change. Mol Ecol 16:619–627

Chow S, Hazama K (1998) Universal primer for S7 ribosomal protein gene intron in fish. Mol Ecol 7:1255–1256

Clayton JW, Tretiak DN (1972) Amine-citrate buffers for pH control in starch gel electrophoresis. J Fish Res Board Can 29:1169–1172

Cockerham CC, Weir BS (1977) Digenic descent measures for finite populations. Genet Res 30:121–147

Dallas JF, Dod B, Boursot P, Prager EM, Bonhomme F (1995) Population subdivision and gene flow in Danish house mice. Mol Ecol 4:311–320

Durand JD, Persat H, Bouvet Y (1999) Phylogeography and postglacial dispersion of the chub (*Leuciscus cephalus*) in Europe. Mol Ecol 8:989–997

Ehlers J, Eissmann L, Lippstreu L, Stephan H-J, Wansa S (2004) Pleistocene glaciations of Northern Germany. In: Ehlers J, Gibbard PL (eds) Quaternary glaciations—extent and chronology. Elsevier B.V., Amsterdam, pp 135–146

Epifanio JM, Philipp DP (1997) Sources for misclassifying genealogical origins in mixed hybrid populations. J Hered 88:62–65

Falush D, Stephens M, Pritchard JK (2003) Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics 164:1567–1587

Falush D, Stephens M, Pritchard JK (2007) Inference of population structure using multilocus genotype data: dominant markers and null alleles. Mol Ecol Notes 7:574–578

Garnier-Géré P, Dillmann C (1992) A computer program for testing pairwise linkage disequilibria in subdivided populations. J Hered 83:239

Guo SW, Thompson EA (1992) Performing the exact test of Hardy–Weinberg proportion for multiple alleles. Biometrics 48:361–372

Haldane JBS (1954) An exact test for randomness of mating. J Genet 52:631–635

Hendry AP, Vamosi SM, Latham SJ, Heilbuth JC, Day T (2000a) Questioning species realities. Conserv Genet 1:67–76

Hendry AP, Wenburg JK, Bentzen P, Volk EC, Quinn TP (2000b) Rapid evolution of reproductive isolation in the wild: evidence from introduced salmon. Science 290:516–518

Hewitt GM (2004) Genetic consequences of climatic changes in the Quaternary. Phil Trans R Soc Lon B 359:183–195

Hofreiter M, Serre D, Rohland N, Rabeder G, Nagel D, Conard N, Münzel S, Pääbo S (2004) Lack of phylogeography in European mammals before the last glaciation. Proc Natl Acad Sci USA 101:12963–12968

Jahns S (2000) Late-glacial and Holocene woodland dynamics and land-use history of the Lower Oder valley, northeastern Germany, based on two, AMS 14C dated, pollen profiles. Veg Hist Archaeobot 9:111–123

Knowles LL (2001) Did the Pleistocene glaciations promote divergence? Tests of explicit refugial models in montane grasshoppers. Mol Ecol 10:691–701

Kohlmann K, Kersten P (1998) Enzyme variability in a wild population of tench (*Tinca tinca*). Pol Arch Hydrobiol 45:303–310

Kohlmann K, Kersten P (2006) Microsatellite loci in tench: isolation and variability in a test population. Aquac Int 14:3–7

Kohlmann K, Kersten P, Flajšhans M (2007) Comparison of microsatellite variability in wild and cultured tench (*Tinca tinca*). Aquaculture 272S1:S147–S151

Kohlmann K, Kersten P, Panicz P, Memiş D, Flajšhans M (2009) Genetic variability and differentiation of wild and cultured tench populations inferred from microsatellite loci. Rev Fish Biol Fisher. doi:10.1007/s11160-009-9138-x

Kotlík P, Berrebi P (2001) Phylogeography of the barbel (*Barbus barbus*) assessed by mitochondrial DNA variation. Mol Ecol 10:2177–2185

Kotlík P, Bogutskaya NG, Ekmekçi FG (2004) Circum Black Sea phylogeography of *Barbus* freshwater fishes: divergence in the Pontic glacial refugium. Mol Ecol 13:87–95

Lajbner Z, Linhart O, Kotlik P (2007) Molecular phylogeography of the tench *Tinca tinca* (Linnaeus, 1758). In: Buj I, Zanella L, Mrakovcic M (eds) The 12th European congress of ichthyology, book of abstracts, Cavtat, 2007, p 35

Lajbner Z, Šlechtová V, Šlechta V, Švátora M, Berrebi P, Kotlík P (2009) Rare and asymmetrical hybridization of the endemic *Barbus carpathicus* with its widespread congener *B. barbus*. J Fish Biol 74:418–436

Machordom A, Doadrio I (2001) Evidence of a cenozoic Betic-Kabilian connection based on freshwater fish phylogeography (*Luciobarbus*, Cyprinidae). Mol Phylogenet Evol 18:252–263

Mayr E (1942) Systematics and the origin of species. Harvard University Press, Cambridge

Mayr E (1963) Animal species and evolution. Harvard University Press, Cambridge

McCune A, Lovejoy NR (1998) The relative rate of sympatric and allopatric speciation in fishes. In: Howard DJ, Berlocher SH (eds) Endless forms: species and speciation. Oxford University Press, Oxford, pp 172–185

Mishler BD, Theriot EC (2000) The phylogenetic species concept (sensu Mishler and Theriot): monophyly, apomorphy, and phylogenetic species concepts. In: Wheeler QD, Meier R (eds) Species concepts and phylogenetic theory. A debate. Columbia University Press, NY, pp 44–54

Muscheler R, Kromer B, Björck S, Svensson A, Friedrich M, Kaiser KF, Southon J (2008) Tree rings and ice cores reveal ^{14}C calibration uncertainties during the Younger Dryas. Nat Geosci 1:263–267

Nagylaki T (1976) The evolution of one- and two-locus systems. Genetics 83:583–600

Nagylaki T (1977) The evolution of one- and two-locus systems II. Genetics 85:347–354

Near TJ, Benard MF (2004) Rapid allopatric speciation in logperch darters (Percidae: Percina). Evolution 58:2798–2808

Nei M (1987) Molecular evolutionary genetics. Columbia University Press, New York

Nesbø CL, Fossheim T, Vøllestad LA, Jakobsen KS (1999) Genetic divergence and phylogeographic relationship among European perch (*Perca fluviatilis*) populations reflect glacial refugia and postglacial colonisation. Mol Ecol 8:1387–1404

Nikinmaa M, Waser W (2007) Molecular and cellular studies in evolutionary physiology of natural vertebrate populations: influences of individual variation and genetic components on sampling and measurements. J Exp Biol 210:1847–1857

Page RD, Holmes EC (1998) Molecular evolution: a phylogenetic approach. Blackwell, Oxford

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959

Raymond M, Rousset F (1995) An exact test for population differentiation. Evolution 49:1283–1286

Ridgway GJ, Sherburne SW, Lewis RD (1970) Polymorphism in the esterases of Atlantic herring. Trans Am Fish Soc 99:147–151

Rieseberg LH, Linder RC (1999) Hybrid classification: insights from genetic map-based studies of experimental hybrids. Ecology 80:361–370

Rousset F (2008) Genepop'007: a complete re-implementation of the genepop software for Windows and Linux. Mol Ecol Res 8:103–106

Rousset F, Raymond M (1995) Testing heterozygote excess and deficiency. Genetics 140:1413–1419

Shaklee JB, Allendorf FW, Morizot DC, Whitt GS (1990) Gene nomenclature for the protein-coding loci in fish. Trans Am Fish Soc 119:2–15

Shaw CR, Prasad R (1970) Starch gel electrophoresis of enzymes–a compilation of recipes. Biochem Genet 4:297–320

Šlechtová V, Šlechta V, Valenta M (1995) Genetic protein variability in tench (*Tinca tinca* L.) stocks in Czech republic. Pol Arch Hydrobiol 42:133–140

Städler T, Arunyawat U, Stephan W (2008) Population genetics of speciation in two closely related wild tomatoes (*Solanum* section *Lycopersicon*). Genetics 178:339–350

Templeton AR (2006) Population genetics and microevolutionary theory. Wiley, Hoboken

Touriya A, Rami M, Cattaneo-Berrebi G, Ibanez C, Augros S, Boissin E, Dakkak A, Berrebi P (2003) Primers for EPIC amplification of intron sequences for fish and other vertebrate population genetic studies. BioTechniques 35: 676–678, 680, 682

Vuorinen J (1984) Electrophoretic expression of genetic variation and duplicate gene activity in vandace, *Coregonus albula* (Salmonidae). Hereditas 101:85–96

Waters JM, Rowe DL, Apte S, King TM, Wallis GP, Anderson L, Norris RJ, Craw D, Burridge CP (2007) Geological dates and molecular rates: rapid divergence of rivers and their biotas. Syst Biol 56:271–282

Weir BS (1979) Inferences about linkage disequilibrium. Biometrics 35:235–254

Weir BS (1996) Genetic data analysis II. Sinauer, Sunderland

Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. Evolution 38:1358–1370

Wheeler QD, Platnick NI (2000) The phylogenetic species concept (sensu Wheeler and Platnick). In: Wheeler QD, Meier R (eds) Species concepts and phylogenetic theory. A debate. Columbia University Press, NY, pp 55–69

Zaykin D, Zhivotovsky LA, Weir BS (1995) Exact test for association between alleles at arbitrary number of loci. Genetica 96:169–178