



Genetic differentiation of European grayling populations across the Main, Danube and Elbe drainages in Bavaria

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Analysis of mitochondrial (*ND-1/3/4* genes) and nuclear (*GH-1* gene, four microsatellite loci) DNA markers of European grayling *Thymallus thymallus* populations identified strong genetic differentiation between the Rhine/Main, Elbe and Danube drainages in Bavaria, as a result of both present processes and past history. The Danube and the Main-Elbe group diverged about five times earlier than the populations of Main and Elbe. These data suggest that exchange and transfer of grayling stocking material between these drainages should be avoided, to maintain the genetic diversity and integrity of populations. © 2001 The Fisheries Society of the British Isles

Key words: mitochondrial DNA; growth hormone gene; RFLP; microsatellites; genetic distances; phylogeography.

INTRODUCTION

The European grayling *Thymallus thymallus* L. is a spring-spawning, lake- or stream-dwelling salmonid that is highly appreciated by sports fishermen. The species is distributed from England eastward across France, Germany, Denmark, Scandinavia and Finland to the Ural mountains, and southward to northern Italy, Switzerland and the drainages of Danube and Dniester rivers (Persat *et al.*, 1978). Grayling is not currently in danger of extinction at the European scale, but in many countries it is endangered at the population level due to water-flow regulation, pollution and habitat destruction (Persat, 1996; Herrmann & Bohl, 1999; Stein & Born, 1999). Conservation and management programmes are being developed and these should consider the genetic structure of grayling populations both at the macro- and micro-geographical scales.

In North America there are two disjunct forms of Arctic grayling *Thymallus arcticus* (Pallas) that are significantly divergent in terms of allozyme allele frequencies (Lynch & Vyse, 1979), mitochondrial haplotype frequencies and distinguishing sequence characteristics (Redenbach & Taylor, 1999). In Europe, current knowledge about the population genetic structure and phylogenetic lineages of grayling is still very poor. The first published studies (Bouvet *et al.*, 1990, 1992; Persat & Eppe, 1997) were based on allozyme analyses and were limited to the grayling populations in France. These studies revealed some

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differentiation between the Loire and Rhône populations, low within-population variation, and some degree of subdivision within the Rhône system itself. A recent microsatellite study by Sušnik *et al.* (1999a) revealed that the grayling populations inhabiting the Danube drainage in Slovenia are genetically distinct from the population in the Soča River, which drains to the Adriatic Sea.

In Bavaria, Germany, a conservation and management programme for grayling has been initiated recently (Stein & Born, 1999) and the aim of the present study was to provide baseline genetic data for this programme by estimating the levels of genetic variation and differentiation of Bavarian grayling populations from three major Central European drainage systems: the Danube, the Elbe and the Rhine. For this purpose three different types of DNA markers were applied: (i) mitochondrial DNA (mtDNA) genes that are inherited as a single non-recombining genealogical unit through maternal lines and are therefore useful for analysis of intraspecific phylogeography, (ii) coding single copy nuclear gene (growth hormone 1 gene) that may be affected by natural selection and thus, may reflect local adaptations, and (iii) highly variable nuclear short tandem repeat loci (microsatellites) that are generally considered selectively neutral and therefore most suitable for estimating levels of differentiation and gene flow.

MATERIALS AND METHODS

SAMPLE COLLECTION AND DNA ISOLATION

Samples of fin tissue from grayling were collected in Bavaria, Germany from the drainages of Rhine/Main (one location: R. Sinn—a tributary of the R. Fränkische Saale, sampling location Rieneck, Unterfranken), Elbe (one location: R. Eger—sampling location Marktleuthen, Oberfranken) and Danube (two locations: R. Ramsach—a tributary of the R. Loisach, sampling location Murnau, Oberbayern and R. Sempt—a tributary of the R. Isar, sampling location Berglern, Oberbayern) (Fig. 1). Forty adult individuals ($L_T > 19$ cm) from each population were sampled by electrofishing a river section of *c.* 1–2 km. As an outgroup for phylogenetic analysis of mitochondrial haplotypes, a geographically distant grayling population from R. Selja (Gulf of Finland, the Baltic Sea) was sampled. Total genomic DNA was isolated according to the simplified method of Laird *et al.* (1991).

GENETIC MARKERS

Mitochondrial DNA

Restriction fragment length polymorphism (RFLP) analysis was performed on two polymerase chain reaction (PCR) amplified segments encompassing the NADH-1 dehydrogenase (*ND-1*) and NADH-3,4 dehydrogenase (*ND-3/4*) gene regions. The primer pairs for amplification of these genes were designed by Nielsen *et al.* (1998). The size of the PCR-products for these regions is *c.* 2000 and 2400 base pairs (bp), respectively. Each PCR reaction (total of 50 μ l) was composed of 1 \times PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 1.5 mM MgCl₂, 0.1 mM dNTPs, 0.2 μ M of each primer and 0.5 units of Taq DNA polymerase (MBI-Fermentas). The PCR amplification consisted of an initial denaturing at 95° C for 5 min followed by 35 cycles of denaturing at 95° C for 30 s, annealing at 60° C for 30 s, and extension at 72° C for 1 min. The PCR products were digested with restriction enzymes *AluI*, *HaeIII*, *HinfI*, *HpaII*, *MboI*, *MvaI*, *NciI*, *RsaI* and *TaqI* and the fragments were resolved on 2% agarose or 6% polyacrylamide gels. The size of the restriction fragments was estimated by comparison to a 100 bp size ladder (MBI Fermentas).

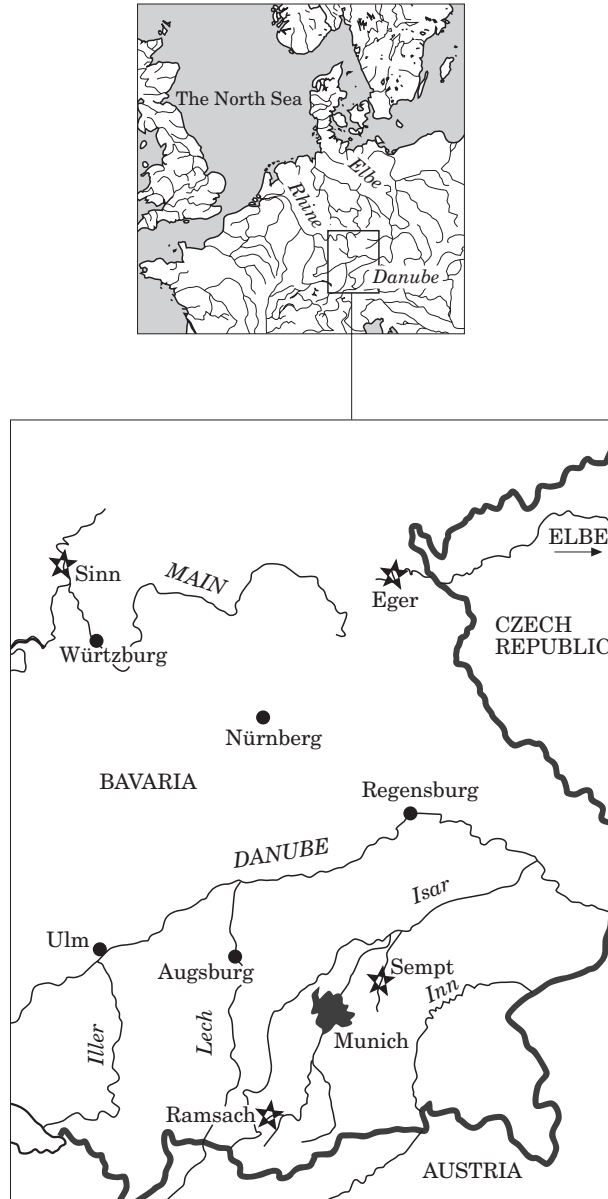


FIG. 1. Sampling locations of grayling in Bavaria (indicated by stars). Grayling were collected from one location in each of the Rhine/Main and the Elbe drainages (Sinn and Eger, respectively) and from two locations in the Danube drainage (Sempt and Ramsach). Grayling were also collected from a location in Estonia (River Selja, which drains into the Gulf of Finland, the Baltic Sea; not shown in the Figure).

Growth hormone 1 gene (GH-1)

RFLP analysis was performed on a PCR amplified segment encompassing the sequence from the first to the fourth exon (c. 1800 bp). A pair of oligonucleotide primers was designed based on the Atlantic salmon *Salmo salar* L. *GH-1* gene sequence (locus *SSGGH* under accession number X61938 in GenBank; Male *et al.*, 1992):

5'-TTGACACAGAACCACCTGCT-3' (positions 853 to 872 according to GenBank)
5'-CTTTGAGGTCGCTGAGCTTC-3' (positions 2496 to 2515, complementary strand).

This primer pair was specific to the *GH-1* gene in grayling and did not cross-amplify the *GH-2* gene. Each PCR reaction (total of 15 μ l) was composed of 1 \times PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 2.0 mM MgCl₂, 0.1 mM dNTPs, 0.2 μ M of each primer and 0.3 units of Taq DNA polymerase (MBI-Fermentas). The PCR amplification consisted of an initial denaturing at 95° C for 5 min followed by 35 cycles of denaturing at 95° C for 30 s, annealing at 60° C for 30 s, and extension at 72° C for 1 min. The PCR products were digested with restriction enzymes *TaqI*, *HaeIII*, *HinfI* and *RsaI*. The fragments were resolved on 8% polyacrylamide gels and visualized using silver staining.

Microsatellites

Twelve microsatellite primer pairs developed for *Oncorhynchus clarki* (Richardson) (Condrey & Bentzen, 1998), *O. nerka* (Walbaum) (Scribner *et al.*, 1996), *O. gorbusha* (Walbaum) (Olsen *et al.*, 1998) and *Salmo trutta* L. (Presa & Guyomard, 1996) were tested for possibility of obtaining polymorphic PCR products in grayling. These loci were selected due to their ability to cross-amplify in the Arctic grayling. Five loci (*Ocl3*, *One7*, *One10*, *Str73*, *Str85*) did not give any amplification products and three loci (*Ocl8*, *Ogo4*, *Ogo5*) turned out to be monomorphic. Thus, only four loci (*Ogo2*, *One2*, *One8* and *One9*) were suitable for analysis of European grayling populations. Each PCR reaction (15 μ l) was composed of 1 \times PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 1.5 mM MgCl₂, 0.1 mM dNTPs, 0.2 μ M of each primer (forward primer fluorescently labelled with TAMRA, FAM, or HEX), and 0.15 units of Taq DNA polymerase (MBI-Fermentas). The PCR amplification consisted of an initial denaturing at 94° C for 3 min followed by 33 cycles of denaturing at 94° C for 15 s, annealing at 60° C for 15 s, and extension at 72° C for 15 s. The genotypes were scored either by electrophoresis in 6% denaturing polyacrylamide gel and subsequent silver staining, or by ABI PRISM 377 Sequencer.

DATA ANALYSIS

For mtDNA data, the genetic relationships among haplotypes were analysed by the distance based Fitch-Margoliash method (using the average number of nucleotide substitutions per site, d_{ij} , as an estimate of evolutionary distance between the haplotypes) and by a maximum likelihood method (using directly the restriction site data matrix) implemented by the programs FITCH and RESTML from the PHYLIP v. 3.572c package (Felsenstein, 1994). Levels of variation within each population were estimated by calculating the unbiased haplotype diversity (h) and nucleotide diversity (π) (Nei, 1987). Nucleotide-sequence divergence among haplotypes (d_{ij}) and the number of net nucleotide substitutions between all pairs of populations (d_A) were calculated for restriction site data according to Nei (1987) using the software package REAP v. 4.0 (McElroy *et al.*, 1991). The matrix of pairwise d_A values was used to construct a neighbour-joining (NJ) dendrogram showing relationships among populations, using the NEIGHBOR program from the PHYLIP v. 3.572c package (Felsenstein, 1994). Pairwise exact tests for heterogeneity in haplotype frequencies between the populations were performed according to Raymond & Rousset (1995a) using the Arlequin v. 1.1 computer programme (Schneider *et al.*, 1997). Genetic differentiation among populations was quantified by analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992) within the Arlequin v. 1.1 package. The matrix of d_{ij} values was incorporated into the analysis as information on DNA haplotype divergence. F_{ST} values and their significance were calculated for all pair-wise comparisons of populations by the Arlequin v. 1.1 package.

For nuclear DNA markers, GENEPOP v. 3.1 (Raymond & Rousset, 1995b) programme was used to generate allele frequency tables, to calculate F_{ST} values, to test genotypic distribution for conformance to Hardy-Weinberg expectations, to test loci for genotypic disequilibrium and to estimate the significance of allelic differentiation between population pairs. All probability tests were based on the Markov chain method (Guo & Thompson, 1992; Raymond & Rousset, 1995a). The populations were tested for

recent reduction of their effective population size by using Wilcoxon sign-rank test as implemented in the BOTTLENECK computer programme, assuming the two-phase model of mutation (with 5% multi-step changes and variance of 12) for microsatellite loci (Piry *et al.*, 1999). Genetic relationships between individuals were estimated based on their multiple microsatellite locus genotype using the shared allele distance, D_{AS} (Bowcock *et al.*, 1994) and the individuals were clustered by NJ method using the program NEIGHBOR from PHYLIP v. 3.572c package (Felsenstein, 1994). An assignment test, based on the Bayesian method (Cornuet *et al.*, 1999), was applied to assess interpopulation dispersal of individuals using the computer program GeneClass v. 1.0.02 (Piry & Cornuet, 1999). Genetic relationships between populations were studied using the Nei *et al.* (1983) D_A distance that was calculated by the computer program DISPAN (Ota, 1993). This measure was chosen because it is independent of the mutation models (Nei, 1987) and superior to other distance measures in correct tree topology construction using microsatellites (Takezaki & Nei, 1996). The matrix of D_A values was used to construct a NJ dendrogram showing relationships among populations, using the PHYLIP v. 3.572c package (Felsenstein, 1994). Bootstrap test (1000 replications) for the obtained tree was performed by the DISPAN program (Ota, 1993).

RESULTS

MITOCHONDRIAL GENES

Polymorphism within the *ND-1* and *ND-3/4* gene regions was revealed by 7 and 8 restriction enzymes, respectively (Appendices I and II). Altogether six different composite haplotypes were identified among the Bavarian grayling populations (Table I). The highest number of haplotypes (4) and consequently the highest diversity values were revealed in the Danube/Sempt population, followed by the Main/Sinn (3 haplotypes), Danube/Ramsach (2 haplotypes) and the monomorphic Elbe/Eger population (Table I). For comparison, a geographically distant grayling population from Estonia (Selja River) was analysed and two additional composite haplotypes were found (the fragment patterns and approximate sizes of the fragments are available from the corresponding author on request). Both distance based and maximum likelihood phylogenetic inference methods clustered the haplotypes into three distinct groups with high bootstrap support (Fig. 2). The average pairwise inter-group divergence (d_{ij}) ranged from 2% (between group I and III) to 3.4% (between group I and II), while within groups the between-haplotype divergence values ranged from 0.14% (between haplotypes 5 and 6, corresponds to a single restriction site difference) to 0.83% (between haplotypes 2 and 3 and between haplotypes 2 and 4, corresponds to eight restriction site differences). Group I contained haplotypes 1 to 4 that were observed in all three Bavarian drainages. However, haplotype 1 dominated in the Main/Sinn population and was not detected in any other population, haplotype 2 was fixed in Elbe/Eger, but was also present in Main/Sinn and haplotypes 3 and 4 occurred only in the Danube drainage at a low frequency (Table I). Group II (haplotypes 5 and 6) was confined to the Danube drainage with the exception of a single individual from Main/Sinn and group III (haplotypes 7 and 8) was confined to Estonia. As a result, 85% of the total mtDNA variation in Bavarian grayling populations was due to the among populations component, indicating a very high level of differentiation between the drainages.

The distance matrix of net interpopulation nucleotide divergence (d_A) was used to construct a NJ tree relating the four populations studied (Fig. 3). The

TABLE I. Haplotype frequencies at the *ND-1/ND-3,4* and *GH-1* genes (allele frequencies for the microsatellite loci are available from the corresponding author on request), sample size (n), number of alleles/haplotypes (A), expected (H_E) and observed (H_O) heterozygosity (haplotype, h , and nucleotide, π , diversity for mtDNA), and Hardy-Weinberg exact test probability (P_{HW}) values in Bavarian grayling populations. Composite haplotypes of the mitochondrial genes are designated by numbers 1–6 and by capital letters (as defined in Appendices I and II) in the following order: *ND-1* (*HaeIII*, *HinfI*, *HpaII*, *NciI*, *MboI*, *MvaI*, *RsaI*)/*ND-3,4* (*AluI*, *HaeIII*, *HinfI*, *NciI*, *MboI*, *MvaI*, *RsaI*, *TaqI*)

Gene allele/haplotype	Parameter	Main/Sinn	Elbe/Eger	Danube	
				Ramsach	Sempt
<i>mtDNA ND-1/ND-3,4</i>					
1 (BBBACBB/BBACAAAB)		0.875	0.000	0.000	0.000
2 (ABBAABB/BBABAABB)		0.100	1.000	0.000	0.000
3 (BCAAABB/BBACAAAB)		0.000	0.000	0.025	0.053
4 (ACBBCBB/BBACAAAB)		0.000	0.000	0.000	0.079
5 (CBBABAA/AABABBCA)		0.025	0.000	0.975	0.421
6 (CABABAA/AABABBCA)		0.000	0.000	0.000	0.447
	n	40	40	40	38
	A	3	1	2	4
	h	0.23	0.00	0.05	0.63
	π	0.0046	0.0000	0.0024	0.0130
<i>GH-1</i>					
a		0.782	0.667	1.000	1.000
b		0.218	0.333	0.000	0.000
	n	39	39	40	40
	A	2	2	1	1
	H_E	0.345	0.450	—	—
	H_O	0.282	0.308	—	—
	P_{HW}	n.s.	n.s.	—	—
<i>Ogo2</i>					
	n	40	40	40	40
	A	12	7	7	8
	H_E	0.845	0.736	0.614	0.832
	H_O	0.750	0.725	0.600	0.775
	P_{HW}	n.s.	<0.001	n.s.	<0.05
<i>One2</i>					
	n	38	37	38	40
	A	14	5	6	10
	H_E	0.838	0.765	0.759	0.839
	H_O	0.763	0.541	0.632	0.725
	P_{HW}	n.s.	n.s.	n.s.	n.s.
<i>One8</i>					
	n	39	40	38	39
	A	11	2	8	21
	H_E	0.749	0.444	0.812	0.905
	H_O	0.744	0.400	0.763	0.846
	P_{HW}	n.s.	n.s.	n.s.	n.s.
<i>One9</i>					
	n	40	40	38	36
	A	4	2	3	4
	H_E	0.421	0.119	0.520	0.554
	H_O	0.450	0.125	0.605	0.556
	P_{HW}	n.s.	n.s.	n.s.	n.s.
<i>Over all microsatellite loci</i>					
	A	10.25	4.0	6.0	10.75
	H_E	0.713	0.516	0.677	0.782
	H_O	0.677	0.448	0.650	0.725
	P_{HW}	n.s.	<0.01	n.s.	n.s.

n.s., Not significant.

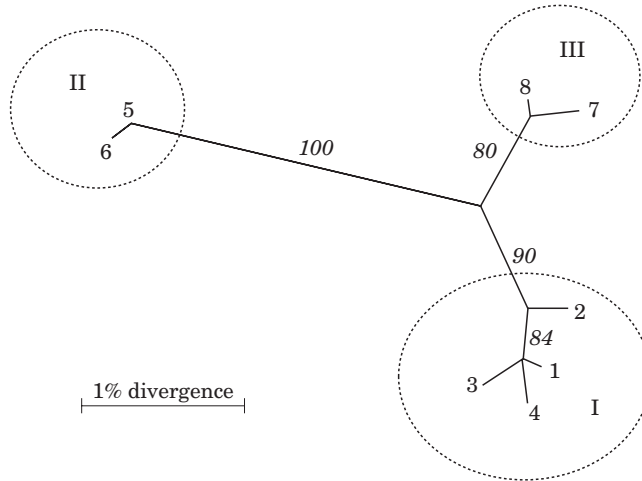


FIG. 2. Unrooted network of grayling mtDNA haplotypes estimated by the Fitch-Margoliash method using the average number of nucleotide substitutions per site (d_{ij}) as an estimate of evolutionary distance between the haplotypes. The composite haplotypes are designated by numbers (from 1 to 8) according to Table III (except the Estonian haplotypes 7 and 8) and the different haplotype groups (delineated by dotted ellipses) are indicated by Roman numbers (I–III). The bootstrap values were obtained from the maximum likelihood tree and only values for nodes supported by at least 50% are shown.

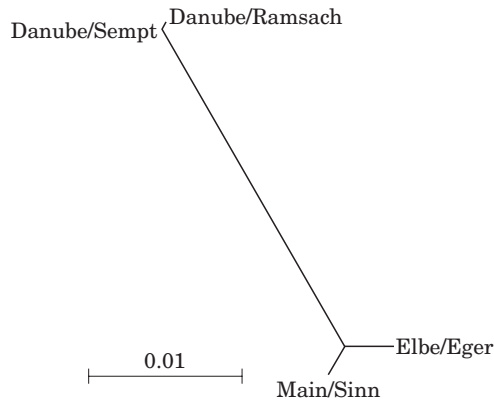


FIG. 3. Unrooted Neighbour-Joining network of grayling populations based on the estimated number of net nucleotide substitutions between all pairs of populations (d_A) at the mitochondrial *ND-1* and *ND-3/4* genes. The tree based on microsatellites and the *GH-1* gene showed identical topology with 100% bootstrap support.

populations clustered into two highly distinct groups (net nucleotide divergence between clusters 2%), reflecting the distribution of phylogenetic groups of mtDNA haplotypes: (i) Danube drainage populations and (ii) Main and Elbe populations. Significant differentiation was observed also within each cluster as the differences of haplotype frequencies were highly significant ($P < 0.001$) in all pair-wise comparisons of populations. The F_{ST} -values were very high for all between-drainages comparisons, but on an average about ten times lower between the two Danube populations (Table II).

TABLE II. Pair-wise estimates of differentiation (F_{ST}) for the mitochondrial genes *ND-1* and *ND-3/4* (above the diagonal) and for the microsatellite loci (below the diagonal)

	Main/Sinn	Elbe/Eger	Danube/Ramsach	Danube/Sempt
Main/Sinn	—	0.788***	0.927***	0.800***
Elbe/Eger	0.352***	—	0.972***	0.850***
Danube/Ramsach	0.282***	0.402***	—	0.090*
Danube/Sempt	0.219***	0.330***	0.086***	—

*** $P < 0.001$.* $P < 0.05$.

NUCLEAR DNA MARKERS

Digestion of the PCR amplified *GH-1* gene region by restriction enzymes revealed length polymorphism (insertion/deletion of at least several nucleotides) within one of the fragments. This polymorphic fragment was the second largest for *HaeIII* and *RsaI*, the third largest for *TaqI* and the fourth largest for *HinfI*. In addition to the length polymorphism, *HinfI* cutting detected restriction site polymorphism within the largest fragment. Given the relatively short distance between the two polymorphic sites, recombination is not a likely factor in generating the genotypes and thus their linkage configuration along the gene can be considered as a haplotype of a single co-dominant locus. Linkage configuration of specific haplotypes was deduced from homozygous individuals. The absence of *HinfI* restriction site was always observed together with the larger size of the 4th fragment, while the presence of the restriction site co-occurred with the smaller 4th fragment. The corresponding composite haplotypes were designated as *GH-1a* and *GH-1b*, respectively. Both haplotypes were detected in the Main/Sinn and the Elbe/Eger populations, but the Danubian populations were fixed for the haplotype *GH-1a* (Table I). The distribution of genotypes in the Main and the Elbe populations corresponded to Hardy-Weinberg expectations (HWE) and the distribution of haplotype frequencies was homogenous between them ($P > 0.05$). A significant deficit of heterozygotes was detected in the Elbe/Eger population ($P < 0.01$).

All four microsatellite loci were in linkage equilibrium ($P > 0.05$), i.e. the genotypes segregated independently at different loci. Variation, expressed as an average number of alleles per locus, was highest in the Danube/Sempt and the Main/Sinn populations (10.75 and 10.25, respectively) and lowest (4.0) in the Elbe/Eger (Table I). The average observed (H_O) and expected (H_E) heterozygosities were also lowest in the Elbe/Eger compared with the other populations (Table I). Significant deviation from HWE was found in two out of 16 probability tests (Table I). After applying a Bonferroni-type correction, only one probability test was significant (for *Ogo2* in Elbe/Eger). However, the multilocus by population test for heterozygote deficit was still significant ($P < 0.001$) for the Elbe/Eger population due to deficit of observed heterozygotes at loci *Ogo2* and *One2*. The Wilcoxon's sign-rank test could not detect excess of heterozygosity in any populations, i.e. the H_E values were not significantly larger ($P > 0.05$) than

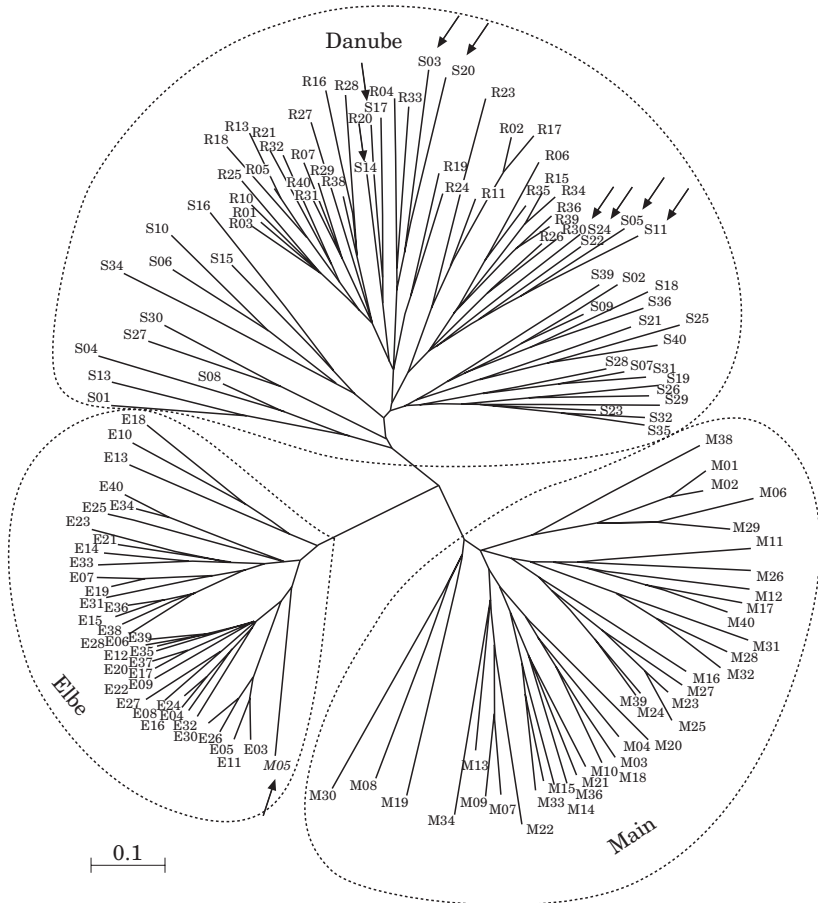


FIG. 4. Unrooted Neighbour-Joining network of grayling individuals based on the shared allele distance, D_{AS} . The misclustered individuals are indicated by arrows.

the heterozygosity expected at mutation-drift equilibrium, indicating that the studied populations have not experienced severe reduction in their effective population size recently.

The differences in microsatellite allele frequencies were highly significant ($P < 0.001$) at all loci for all pairwise comparisons of populations. Different alleles predominated in different drainages and only 5.3% of alleles over all loci were shared among them, while 23.9, 25.7 and 6.8% of alleles were shared between Main and Elbe, Main and Danube, and Elbe and Danube, respectively. Pair-wise estimates of F_{ST} between populations indicated significant differentiation between the three drainages (overall $F_{ST} = 0.30$) and on an average four times lower (but still significant) differentiation within the Danube drainage (Table II). The unrooted NJ tree built from the matrix of pairwise D_{AS} distances among all 160 specimens clustered grayling individuals into three major clusters corresponding to the three drainages, except for a single individual (Main/Sinn no. 05) that clustered together with the Elbe/Eger group (Fig. 4). However the two Danube populations were not so distinct—20% of the R. Sempt graylings

(eight individuals) clustered with the R. Ramsach population. The Bayesian assignment test also assigned individuals to the three drainages with high accuracy (with the exception of the same misassigned Main/Sinn specimen) and could even assign 98.75% of individuals from the two Danubian populations correctly to their population of origin. However, the probability that the individual belongs truly to a given population was rather low (<0.05) for 12.5 and 30% of fish from Ramsach and Sempt, respectively.

For further analysis, the microsatellite and *GH-1* data were pooled. Pair-wise estimates of F_{ST} between populations were almost identical with the estimates based on only microsatellite loci and the topology of the tree based on the D_A distance matrix was fully congruent with the population tree based on the mtDNA data (Fig. 3).

DISCUSSION

Results from the analyses of mitochondrial and nuclear genes were generally congruent. The ranking order of grayling populations based on the estimates of intrapopulation variation (number of alleles/haplotypes, heterozygosity/diversity) coincided for the mtDNA and microsatellite data – the highest variation was observed in the Danube/Sempt population, followed by the Main/Sinn, Danube/Ramsach and Elbe/Eger populations. The least varied Elbe/Eger population exhibited significant deficit of heterozygotes at the *GH-1* gene and at two microsatellite loci. However, the reduction of heterozygote frequencies was not the same for all genes and not all heterozygous genotypes were in deficit. Therefore, the heterozygote deficiency in the Elbe/Eger population is caused more likely by sampling of different sub-populations (Wahlund effect) or related individuals (Allendorf-Phelps effect) than by inbreeding. The estimates of intrapopulation variation do not correlate well with the population density (estimated by the catch and release method). For example, the river with the highest density was Danube/Ramsach (12.9 adult specimens ($L_T > 19$ cm) per 100 m²) followed by the Elbe/Eger (3.5) and the lowest densities being in the Main/Sinn and the Danube/Sempt (1.5 and 1.3, respectively). This is not in agreement with the general tendency for a positive correlation between genetic variation and population size within species (Frankham, 1996; Hänfling & Brandl, 1998a) and may be related to historical events, e.g. population bottlenecks. The test for heterozygosity excess (Luikart & Cornuet, 1998) could not identify any populations that had experienced recently a severe reduction in their effective population size but the power of the bottleneck test is rather low with only four microsatellite loci. The higher variation of the Sempt population compared with the Ramsach within the Danube drainage can be explained by stocking of graylings from other tributaries of the R. Isar system into the Sempt River during the last 40 years, while the Ramsach has been stocked by fingerlings of exclusively local origin since 1995.

Both mitochondrial and nuclear data sets identified very strong genetic differentiation of Bavarian grayling populations between the Main, Elbe and Danube drainages, illustrated by highly significant heterogeneity of allele/haplotype frequencies, high number of private microsatellite alleles, high F_{ST} values and high accuracy of individual assignments. In contrast, differentiation

between the two grayling populations within the Danube drainage was rather low and exchange of migrants has been rather common historically as indicated by high percentage of misclustered individuals and low probability of individual assignments. The number of effective migrants per generation ($N_e m$), inferred indirectly from the F_{ST} -values, was <1 between drainages and >1 between the two sites within the Danube drainage. However, the accuracy of these estimates is very low because it is very likely that real populations violate the assumptions underlying the translation of F_{ST} to $N_e m$ (Waples, 1998; Whitlock & McCauley, 1999) and the estimates do not necessarily reflect levels of contemporary gene flow (Bossart & Prowell, 1998). For example, both Danubian populations belong to the Isar River system and geographically are rather close to each other. However, migration between these rivers has been impossible due to numerous hydropower plants since at least the 1920s and no transfer of stocking material between them has been practised. Thus, the isolation period of Ramsach and Sempt populations has been too short for developing any significant genetic differentiation and the low F_{ST} value (or accordingly the high $N_e m$ value) does not imply current gene flow. A more detailed and accurate description of the local differentiation pattern of grayling in Bavaria can be obtained by analysing additional populations within drainages with a higher number of microsatellite loci. Grayling-specific microsatellite loci have been isolated recently (Snoj *et al.*, 1999; Sušnik *et al.*, 1999a, b) and Koskinen & Primmer (1999) have reported additional salmonid primer pairs that cross-amplify European grayling microsatellites.

Both mitochondrial and nuclear DNA based estimates of genetic distances suggest that the Main and Elbe grayling populations are related more closely to each other than to the Danubian populations and that splitting occurred first between the Danube and the Main-Elbe group and then between Main and Elbe. The relative time since divergence of the Main and the Elbe populations is about five times shorter than between the populations from Main and Danube or from Elbe and Danube, as estimated by the mtDNA RFLP data. This is not congruent with the estimates based on the D_A distances of nuclear markers, but the correlation of this measure with evolutionary time is low (Nei, 1987) and the estimates are likely to be biased upwards with fewer than 50 to 100 fish per sample (Ruzzante, 1998). Takezaki & Nei (1996) suggested that for estimating evolutionary times the $(\delta\mu)^2$ distance of Goldstein *et al.* (1995) is more appropriate when using microsatellites, but $(\delta\mu)^2$ is based strictly on a stepwise mutation model and no allele size constraints, assumptions that may not be valid for the studied microsatellite loci.

The mtDNA RFLP data on *ND-1* and *ND-3/4* gene regions are in good agreement with a recent sequencing study of the *ND-5* gene among European grayling populations which also revealed three distinct mitochondrial haplotype groups with average between-group divergence values almost identical to present data (Koskinen *et al.*, 2000). The *ND-5* gene study also included the Elbe/Eger, the Danube/Ramsach and the Estonian Selja River populations and the Elbe/Eger population shared identical or closely related haplotypes with the Polish and western Fennoscandian grayling populations (haplotype group I according to present designation), while the Estonian haplotypes grouped together with the Finnish and northwestern Russian haplotypes (haplotype group III). The average

divergence between these two haplotype groups was significantly smaller (1.11 and 1.98% based on the sequencing and present RFLP data, respectively) than between them and the haplotype group II, prevailing in the Danube drainage (3.30 and 3.59% based on the sequencing data, 3.1 and 3.4% based on present RFLP data for between-group comparisons I–II and III–II, respectively). However, present results demonstrate that the Danubian populations possess a mixture of haplotypes from different phylogenetic clades—besides the typical Danubian haplotypes 5 and 6 (group II) there occur less frequent haplotypes 3 and 4 that are phylogenetically close to the haplotypes 1 and 2 from the Main and the Elbe (group I). A rather similar situation has been detected for European perch *Perca fluviatilis* L. by Nesbø *et al.* (1999)—the western European mitochondrial haplotypes that dominate in Vistula, Elbe, Rhine, Rhône and Thames also occur in the upper Danube along with the highly divergent ancient Danubian haplotypes. Their explanation was that the western European drainages were recolonized by perch from a common western glacial refugium (the exact location of which was unclear) and this group intergraded subsequently with the Danubian group which did not contribute to the most recent recolonization of northern glaciated areas. Another widely distributed European fish species, the chub *Leuciscus cephalus* L., also exhibits a pattern of genetic relationships similar to the grayling—the Main and the Elbe populations cluster together and are distant from the Danube populations (Hänfling & Brandl, 1998b). Based on the distribution of chub mtDNA haplotypes in Europe, Durand *et al.* (1999) proposed a scenario in which the Rhine and Elbe drainages were recolonized from a Danubian refuge in two steps—the Danubian lineage entered western Europe (Rhine-Rhône-Loire drainages) during the Riss-Würm interglacial period (about 100 000 years BP) and survived the last glaciation there before colonizing the German drainages up to the Elbe during the Holocene (about 7000 years BP). In contrast to chub and grayling, the bullhead *Cottus gobio* L., populations from the Elbe and the Danube are more closely related and differ significantly from the Main populations (Hänfling & Brandl, 1998c). According to the estimates based on allozyme data, the Main and the Elbe-Danube bullhead stocks diverged between 300 000 and 600 000 years BP, while the Danube and Elbe stocks diverged between 100 000 to 200 000 years BP (Hänfling & Brandl, 1998c). Englbrecht *et al.* (2000) studied bullhead populations from the same area by mitochondrial D-loop sequencing and revealed that the average mean pairwise distance between the Main, Elbe and Upper Danube drainages was 0.7% that corresponds to the divergence time between 350 000 to 700 000 years BP depending on the applied mutation rate, suggesting their Pleistocene origin.

No attempt will be made to provide hereby any absolute divergence time estimates for the grayling, because there are currently no good reference points for calibrating the mtDNA molecular clock. However, it seems reasonable to presume that the Danubian and the Main/Elbe lineages diverged already before the Würm glaciation period (about 70 000 to 100 000 years BP) or maybe even before the Riss glaciation (about 150 000 to 300 000 years BP), as suggested by the deep divergence between the mitochondrial haplotype groups I and II. These two lineages may have been in secondary contact later, as indicated by the mixture of mitochondrial haplotypes from different phylogenetic lineages in the

upper Danube. Occurrence of two *GH-I* gene haplotypes in the Main/Sinn and the Elbe/Eger populations in contrast to a single haplotype in the upper Danube provides further support for the hypothesis that the Danubian populations did not contribute significantly to the most recent postglacial recolonization of the northern river systems. Probably the Main/Elbe group could have survived the glaciation(s) in the part of Central Europe lying between the northern and the Alpine ice caps (Bănărescu, 1992) or in a refugium westward, on the territory of present day France and the isolation of the Main and the Elbe populations is relatively recent. However, it should be stressed that the present study is based on only four populations from three drainages in an area that covers only a small section of the species natural distribution and therefore, further studies involving sampling of populations from a much broader area and sequencing of the mtDNA haplotypes are necessary for clarifying the phylogeny and colonization history of grayling in Europe.

Present data on mtDNA in conjunction with nuclear markers demonstrated unequivocally significant genetic differentiation between the grayling populations from the three large river drainages of Rhine/Main, Elbe and Danube that is the result of both present processes and past history. For this reason, exchange or transfer of grayling stocking material between these drainages should be avoided, to maintain the historically established genetic diversity and integrity of populations.

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