

Discriminating the impact of recent human mediated stock transfer from historical gene flow on genetic structure of European grayling *Thymallus thymallus* L.

B. GUM*†, R. GROSS‡ AND R. KUEHN*

*Wildlife Biology and Management Unit, Department for Ecosystem and Landscape Management, TU Munich-Weihenstephan, D-85354 Freising, Germany and ‡Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, EE-51014 Tartu, Estonia

Microsatellite markers were first used to partition individuals of European grayling *Thymallus thymallus*, from the Danube, Rhine and Main, and Elbe drainage systems into subpopulations and to estimate individual immigrant ancestries over the last few generations. Subsequently, the studied populations were 'purged' from recent immigrants and the proportions of evolutionary lineages within the 'purged' populations were re-estimated by applying mtDNA markers. The results confirmed a high level of admixture of the divergent mtDNA lineages (*i.e.* natural secondary contact) in populations sampled at the contact zones of the drainages. In addition, a substantial amount of introgression was observed for several populations that were known to be affected by stocking of European grayling from different origins.

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INTRODUCTION

Large-scale phylogeographical studies carried out over the last decade have identified several distinct glacial refugia for the northern hemisphere fish species where populations could diverge genetically in allopatry (Bernatchez & Wilson, 1998; Englbrecht *et al.*, 2000; Bernatchez, 2001; Kotlík *et al.*, 2004). Also, several studies have identified specific regions where these genetically distinct lineages naturally came into secondary contact after the last glaciation (Froufe *et al.*, 2003; Taylor, 2004; Gum *et al.*, 2005). The dynamics of these naturally occurring genetic interactions have been further complicated by the introgression of exogenous gene pools through human mediated non-native stock transfers or accidental escapes of farmed fishes which may present a considerable threat to the genetic diversity and integrity of native fish stocks (Hindar *et al.*, 1991; Taylor, 1991; Hansen *et al.*, 1995, 2001a; Fleming *et al.*, 2000;

†Author to whom correspondence should be addressed. Tel.: +49 8161 714610; fax: +49 8161 714615; email: gum@wzw.tum.de

Sušnik *et al.*, 2004; Vasemägi *et al.*, 2005), and also can result in the transfer of diseases and parasites (Jonsson, 1997). Given the fact that the introduction and stocking of non-native fishes still takes place in many countries, it is necessary to apply and further develop the appropriate tools to (1) distinguish between natural and anthropogenic caused admixture of distinct genetic lineages and (2) determine the level of introgression of stocked into native gene pools. These results can then be used for phylogeographical inferences and are essential for prioritizing conservation interventions and the setting of conservation units (Allendorf *et al.*, 2001). The exclusion of certain 'stocked' populations from restoration efforts or conservation measures, however, may not always be justified, because the long-term success of many stocking activities cannot be predicted *a priori* (Thorfve, 2002; Ruzzante *et al.*, 2004), and indeed, past stocking practices have not always led to introgression and loss of a formerly distinct population (Mezzer & Largiader, 2001; Hansen, 2002; Ruzzante *et al.*, 2004).

Today molecular genetics and the appropriate statistical analyses provide the opportunity to discriminate between long-term gene flow (historical secondary contact) from recent immigration (stocking). As computational constraints have been largely overcome over the past decade (Beaumont & Rannala, 2004), the presently available methods accommodate recent population expansions, non-symmetrical migration and other complexities that are typical of real biological populations (Beerli & Felsenstein, 2001; Wilson & Rannala, 2003). In particular, several new Bayesian methods have been proposed to allow the combined inference of both the partitioning of individuals into subpopulations (Pritchard *et al.*, 2000; STRUCTURE), and the assignment of individual migrant ancestries of individuals into subpopulations (Dawson & Belkhir, 2001; Wilson & Rannala, 2003; BAYESASS). These methods generally require fewer assumptions than estimators of long-term gene flow and can be legitimately applied to non-stationary populations that are far from genetic equilibrium. In addition, recent simulation studies show that the expected accuracy of Bayesian inference is higher when directly compared to competing methods such as maximum likelihood (Beerli, 2006).

For the European grayling *Thymallus thymallus* (L.), stocking and supportive breeding programmes increasingly have been carried out during the last 20 years throughout central Europe with the primary aim of counteracting the strong decline in natural population densities (Baars *et al.*, 2001). The species is critically endangered at the population level (Gross *et al.*, 2001; Gum *et al.*, 2003) due to its behaviour and habitat preference, which make it particularly vulnerable to predation by piscivorous birds (Northcote, 1995; Staub *et al.*, 1998). A natural contact zone for three highly divergent evolutionary lineages of European grayling exists in Bavaria (Gum *et al.*, 2005), where the headwaters of the major central European drainages of the Danube, Rhine and Main, and Elbe are closely juxtaposed. In these river systems, fishery managers have usually used juvenile fish (0+ to 1+ years; in recent times 1+ to 2+ years) from spawners of the same river stretch to support the depleted stocks (supportive breeding). Fish originating from different river or drainage systems, however, have also been used occasionally for enhancement and this may have affected the genetic structure of native populations. Several populations in this

region have been shown to be genetically admixed (Gum *et al.*, 2005), but it remains to be clarified whether this is the result of natural (historical gene flow and secondary contact) or anthropogenic (stock transfer) factors.

In this study the Bayesian methods as proposed by Pritchard *et al.* (2000) and Wilson & Rannala (2003) were applied in order to discriminate between historical secondary contact and recent stocking events. Microsatellite markers were first used to partition individuals into subpopulations and to estimate the posterior probability distributions of individual immigrant ancestries over the last few generations. Then the studied populations were 'purged' from recent immigrants and the proportions of evolutionary lineages within the 'purged' populations were re-estimated by applying mtDNA markers. The presence of multiple mtDNA lineages in these 'purged' populations should more reliably indicate the admixture due to historical secondary contact.

SAMPLE COLLECTION AND STOCKING HISTORY

A total of 735 individuals of European grayling originating from 26 locations across the drainages of the Danube, Rhine and Main, and Elbe from Bavaria, Germany, Austria and the Czech Republic were caught from the wild primarily by electrofishing, or were provided as fin clips preserved in 96% alcohol (Table I and Fig. 1). In addition to the sampling scheme of Gum *et al.* (2005), five new populations were included: Iller, Ilm, Isar, Saalach and Weißer Main. Populations were chosen based on different status of management regarding stocking or supportive breeding actions described below. For the majority of locations sampling was carried out during the European grayling spawning time in March and April in a single year. In order to avoid collection of potential distinct spawning populations the sampled river stretches generally did not exceed 1–2 km (European grayling spawners usually congregate close to their spawning grounds). The sex ratio of adult specimen was *c.* 1:1 in all samples, except for the Ilm population where only female spawners could be obtained. Concerning the decline of European grayling over the last 20 years, the Isar and Iller Rivers have been among the most heavily affected ones (Baars, 2000). Here, a stretch of *c.* 6–8 km had to be sampled to catch a representative number of adult and juvenile European grayling including different year classes. Due to the limited number of individuals obtained from the Rivers Isar, Leinleiter and Schwarzer Regen in a single year, samples from two consecutive years were collected and combined (Table I). Six of the 23 individuals from the River Inn (Innsbruck, population InA) were caught from another tributary of that area (M. Martys, pers. comm.). Samples from both the Rivers Ammer and Saalach were caught from two closely adjacent river stretches. In order to test for the hypothesis of distinct (spawning) sub-groups present within populations Am, Saa and InA, respectively, individuals originating from these distinct stretches at first were considered as a single population.

Regarding the stocking history of European grayling north of the Alps, in general there was no need for intensive stocking until the decline started *c.* 20 years ago (Baars, 2000; Hanfland, 2002). In addition, Baars *et al.* (2001) pointed out that the establishment of permanent European grayling broodstocks in hatcheries has never been a common practice in Bavaria.

TABLE I. Major river drainage, population name, code, sample size, year of sampling, age composition and management status of European grayling populations

Drainage/ sub-drainage	Population	Code	Geographical position	n	Year of sampling	Age (years)/ year classes	Status
Elbe/Eger	Eger	Ege	50°07' N; 12°00' E	38	1999	Adults/mixed	Wild
Elbe/Eger	Tepla	Tep	50°31' N; 13°20' E	20	2002	Spawners	Hatchery
Elbe/Sä.Saale	Sächsische Saale	Ssa	50°15' N; 11°56' E	20	2000	Adults/mixed	Stocked ²
Elbe/Sä.Saale	Lamitz	Lam	50°14' N; 12°01' E	13	2002	Adults/mixed	Wild
Elbe	Volyuka	Vol	49°16' N; 13°37' E	29	2002	Spawners	Hatchery
Main/Fr.Saale	Fränkische Saale	FrS	50°07' N; 09°56' E	15	2000	Spawners	Wild
Main/Fr.Saale	Sinn	Sin	50°09' N; 09°38' E	39	1999	Adults/mixed	Wild
Main/Wisent	Leinleiter	Lei	49°47' N; 11°11' E	30	1999,2000	Spawners	Stocked ³
Main	Weißer Main	WM	50°07' N; 11°60' E	22	2004	Spawners	Wild
Main	Erf	Erf	49°42' N; 09°16' E	15	2000	Adults/mixed	Wild
Rhine	Bodensee	BoS	47°34' N; 09°36' E	39	2001	All 1+	Hatchery
Rhine	Liechtenstein BK	LiB	47°08' N; 09°31' E	20	2001	Spawners	Wild
Danube/Inn	Inn (Gars)	InG	48°09' N; 12°16' E	50	2000	Spawners	Wild ¹
Danube/Inn	Inn (Innsbruck)	InA	47°04' N; 10°40' E	23	2002	Spawners	Wild ^{1*}
Danube/Inn	Saalach	Saa	47°73' N; 12°88' E	30	2005	Spawners	Stocked ¹
Danube/Inn	Tittmoninger Ache	TiA	48°03' N; 12°46' E	39	1999	Spawners	Stocked ¹
Danube/Isar	Ammer	Am	47°52' N; 11°09' E	36	2000	Spawners	Wild ^{1*}
Danube/Isar	Isar	Isa	48°40' N; 11°73' E	33	2000, 2001	Adults/1+	Stocked ^{1*}
Danube/Isar	Sempt	Sem	48°23' N; 11°55' E	39	1999	Spawners	Stocked ¹
Danube/Isar	Ramsach	Ram	47°40' N; 11°12' E	39	1999	Spawners	Wild ¹
Danube/Iller	Iller	Ile	47°57' N; 10°22' E	34	2005	Adults/mixed	Stocked ^{1*}
Danube/Lech	Lech	Lec	47°49' N; 10°53' E	20	1999	Spawners	Wild ¹
Danube/Naab	Fichteinaab	FiN	49°48' N; 12°09' E	25	2002	Adults/mixed	Wild
Danube/Naab	Waldnaab	WaN	49°47' N; 12°01' E	10	2002	1+ and 2+	Wild
Danube/Regen	Schwarzer Regen	ScR	48°58' N; 13°07' E	32	1999, 2000	Spawners, 0+	Stocked ^{1*}
Danube	Ilm	Ilm	48°40' N; 11°22' E	25	1999	Spawners	Stocked ^{1*}

Status 'stocked', grayling originating from different rivers were stocked at least once in the last decades; 'wild', no human mediated stock transfer known. *Stocking history; ¹supportive breeding; ²single stocking event in 1990 upstream of sampling site with European grayling of Danubian origin; ³re-established 35 years ago from nearby river Wisent.

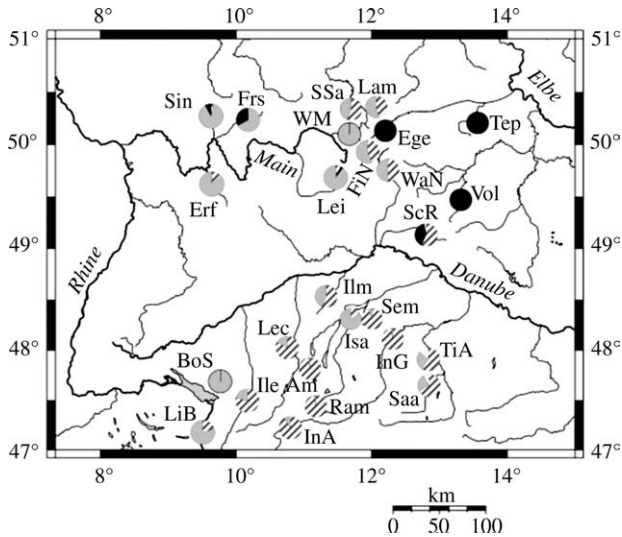


FIG. 1. Map of sampling locations with pie diagrams showing the distribution of major mtDNA lineage frequencies among studied European grayling populations (see Table I): lineages (see Table II) I (○), IIa (◐), IIb (●) and III (▨).

Instead, European grayling spawners are still caught from the wild mostly by electrofishing. When more intensive stocking started at the end of the 1980s the southern tributaries of the Danube (Rivers Inn, Isar, Lech and Iller, and their tributaries) were considerably more affected than the northern Bavarian rivers of the Danube, Main and Elbe drainages. Concerning the Main and Elbe drainages, supportive breeding or stocking actions were reported only in populations Lei and Ssa (see Table I). In this study, several populations from each drainage were included where no human caused stock transfer has been documented or known (indicated as 'wild'; Table I). Supportive breeding by stocking of fingerlings of local origin is known for several Danubian populations: Ram, Am and Lec (since 1995), and InG (since the end of the 1980s). In addition, several populations have also been influenced by stocking of European grayling from different origins: Sem (from different tributaries of the Isar system since 40 years), TiA (regular stocking from Rivers Alz, Inn and Dorfen since the middle of the 1980s), Ilm (several times from different southern Danubian tributaries but also from the Main drainage since 15–20 years), Isa (regular stocking from both local and different origins from Bavaria until 1999, then stopped), Saa (from 2000 to 2003 stocking of offspring from different southern tributaries of the Danube, since 2003 exclusively supportive breeding), ScR (regular stocking of 1+ year fish from a natal broodstock from a local hatchery; Baars, 2000). Data concerning the origins of stocked fish and number of stocking events are based on records of the hatchery of the Bavarian Fisheries Association and personal records of local anglers' associations. It should be noted that especially for the bigger rivers Inn, Saalach, Isar, Lech or Iller downstream migration of stocked European grayling from upstream stretches is known (pers. obs.) and therefore assessment of the stocking

history of sampling sites located downstream has to be considered with caution. Hatchery samples BoS, Tep and Vol stem from wild populations (tributaries to the Alpine Rhine and Elbe, respectively) and were transferred to local hatcheries for the purpose of captive breeding (P. Kotlik & E. Bohl, pers. comm.).

METHODS

DNA ISOLATION AND GENETIC ANALYSIS

Genomic DNA was isolated according to the simplified method of Laird *et al.* (1991). Mitochondrial RFLP analysis was performed on two polymerase chain reaction (PCR) amplified segments encompassing the *NADH-1* (ND-1) and *NADH-5/6* (ND-5/6) gene regions. These mtDNA regions were selected because they allowed, in previous studies, to reveal major evolutionary lineages of *T. thymallus* in Europe (Koskinen *et al.*, 2000; Gum *et al.*, 2005). Details of PCR amplification, RFLP analysis, sequencing mtDNA haplotypes and lineage designation and phylogenetic analysis are given in Gum *et al.* (2005).

A total of 12 microsatellite loci were analysed which have been used in earlier population and phylogeographic genetic studies on European grayling (Gum *et al.*, 2005): *BFRO004*, *BFRO005*, *BFRO006*, *BFRO007*, *BFRO009*, *BFRO010*, *BFRO011*, *F43*, *Ocl8*, *Ogo2*, *One9* and *SSOSL311*. Original references for the microsatellites and details of PCR and genotyping procedures are outlined in Gum *et al.* (2003).

DATA ANALYSIS

For nuclear DNA markers, the FSTAT v. 2.9.3 programme package (Goudet, 2001) was used to calculate allele frequencies and to estimate the expected (H_E) and observed (H_O) heterozygosities, the number of private alleles (A_{pr}) and the allelic richness (A_R). GENEPOP v. 3.3 (Raymond & Rousset, 1995a) was used to test genotypic distributions for conformance to Hardy–Weinberg (HW) expectations, and to test the loci for genotypic disequilibria. All probability tests were based on the Markov chain method (Guo & Thompson, 1992; Raymond & Rousset, 1995b) using 1000 de-memorization steps, 100 batches and 1000 iterations per batch. The data were assessed for the potential genotyping errors, such as null alleles, short allele dominance (large allele dropout), or scoring errors, by using the computer programme MICRO-CHECKER 2.2.3 (van Oosterhout *et al.*, 2006). To better visualize the admixed populations among the drainages of the Danube, Rhine and Main, and Elbe, a multidimensional scaling analysis was performed (Kruskal & Wish, 1977) with STATISTICA v. 6.0 (StatSoft, Inc., Tulsa, OK, U.S.A.) based on the matrix of pair-wise F_{ST} values, which were calculated by FSTAT. This analysis permits the genetic relationships among populations to be represented with minimum loss of information. The optimal number of dimensions (two for the data set) was chosen based on the plot of stress values against different numbers of dimensions.

The presence of intra-population and inter-population genetic structure was tested using the model-based clustering method of Pritchard *et al.* (2000) implemented in STRUCTURE v. 2.1. This analysis was used to (1) assess if there is indication for the presence of multiple gene pools within populations that can be linked to contemporary phenomena or sampling and (2) infer the uppermost hierarchical level of population genetic structure (or clusters, respectively) represented by the whole data set. For the first approach, the Markov chain Monte-Carlo (MCMC) scheme was run with a 'burn-in' period of 50 000 steps and a chain length from 250 000 to 500 000. Multiple runs were performed to assess convergence of $\ln \Pr(X|K)$, and the number of gene pools present within each sampling site was then determined from posterior probabilities of K estimated using a uniform prior on $K = \{1,2,3,4\}$. As recommended by Pritchard *et al.*

(2000), a visual inspection of estimated values of Q (the membership coefficient for each individual into each population) was performed in all cases, *i.e.* when estimates of Q showed a uniform distribution ($Q \simeq K^{-1}$) it was concluded that a value of $K = 1$ was an appropriate model for the data. For inferring the uppermost hierarchical structure of the sampled distinct gene pools an *ad hoc* statistic ΔK (based on the rate of change in the \ln probability of data between successive K values) was used according to Evanno *et al.* (2005). Ten independent runs were performed ('burn-in' 15 000 steps, sampling iterations 150 000, different degrees of admixture allowed and correlated allele frequencies) for each value of K (number of populations) between 1 and 20 [$K \geq 20$ resulted in a strong increase of variance of mean $\ln(K)$]. The most likely value of K is the one where the change of likelihood from one value to the next drops considerably and consecutive values tend to plateau.

Individual immigrant ancestries were estimated by the Bayesian method of Wilson & Rannala (2003), implemented in BAYESASS v. 1.3, which relies on MCMC techniques. One key advantage compared to earlier methods for detecting recent immigrants is that BAYESASS requires fewer assumptions and allows genotype frequencies to deviate from Hardy–Weinberg equilibrium (HWE) proportions within populations (indeed, this is often observed in populations affected by human activities). Optimal programme settings were determined initially in respect to the total likelihood by repeatedly running the software BAYESASS v. 1.3. using different delta values (0,05; 0,10; 0,15) of migration rate and the level of inbreeding (F), respectively. To estimate the posterior probability distribution of parameters, the MCMC chain was run for a total of 3 000 000 iterations, discarding the first 1 000 000 iterations as 'burn-in' (testing iterations of 30 000 000 did not change the overall results). Samples were collected every 2000 iterations. Posterior probabilities of each individual immigrant ancestry were estimated for each population with the aim to identify non-immigrants, first generation immigrants and offspring of immigrants and non-immigrants. In this study the latter group is termed 'second generation immigrants'. It should be noted that the method basically allows more distant migrant ancestries dating back several generations ago to be detected (Wilson & Rannala, 2003; B. Rannala, pers. comm.). The current implementation of BAYESASS, however, does not use information from migrants arriving more than two generations ago to estimate the migration rate (m) (B. Rannala, pers. comm.). In the present data set, the natural migration has been extremely limited between most population pairs over the last century (except for Lam/Ssa, FiN/WaN, Saa/TiA; see Table I) due to numerous impassable weirs and dams within each river system. Therefore, recent immigration rates in the material are expected to primarily reflect stocking effects.

In order to discriminate between the historical and contemporary (*i.e.* stocking) gene flow, only individuals with a posterior probability $>90\%$ of being non-immigrants were used to re-evaluate the proportion of different mtDNA lineages within populations. This should reflect more reliably the level of admixture of divergent mtDNA lineages due to historical secondary contact. In addition, STRUCTURE was used to specifically assess the level of admixing between drainages Danube, Rhine and Main, and Elbe before and after exclusion of the identified immigrants. Individual admixture coefficients for each population were calculated assuming a model of $K = 3$ (representing the uppermost hierarchical structure, *i.e.* the evolutionary lineages) and using the same running parameters as outlined above for $K = \{1 \text{ to } 20\}$.

RESULTS

MTDNA DIVERSITY AND GEOGRAPHICAL DISTRIBUTION OF MTDNA HAPLOTYPES

Previous phylogenetic analyses clustered the mtDNA haplotypes of European grayling into three major lineages I, II and III (Koskinen *et al.*, 2000;

Gum *et al.*, 2005). Lineage I was confined to northern Europe, lineage II to central Europe and lineage III was denoted as the Danubian lineage. Gum *et al.* (2005) suggested a further division of central European lineage II into lineage IIa (predominantly found in Rhine and Main drainage in western Europe), and IIb (predominantly found in the Elbe drainage and in eastern, north-eastern Europe). The geographical distribution of lineages IIa, IIb and III in the studied region and the variable proportions of the lineages within populations are shown in Fig. 1 (see also Table II). Especially in the contact zones of tributaries to the upper Danube, upper Main and Elbe, populations such as FiN, Lam, WaN and Ssa (area of the Fichtelgebirge) or ScR exhibited *c.* 50% of haplotypes belonging to different lineages (Table II). Mitochondrial RFLP analysis revealed three new composite haplotypes (two belonging to lineage IIa and one to lineage III) for populations Ilm (abccaicaabba, designation according to Gum *et al.*, 2005), WM (abcdaeidaebba), and Saa (hcbaadbdbabbha). Sequencing of the ND-5 region of these haplotypes revealed that these new RFLP haplotypes in Ilm and WM were identical to the sequence haplotypes Cw6 and Cw7, respectively (Gum *et al.*, 2005), and the one found in the Saa population resulted in a new ND-5 sequence haplotype designated as DAN05 (GenBank accession number DQ643256). The highest diversity values (Table II) and the highest numbers of haplotypes within the Danubian drainage were found in populations TiA (nine), Saa (seven), ScR (six) and Sem (six), within Rhine and Main in LiB (five) and within the Elbe drainage in Ssa (three) and Lam (three). Populations Ege, Tep, Vol, and WM were fixed for a single haplotype.

MICROSATELLITE DNA VARIATION WITHIN AND BETWEEN SAMPLES

The estimates of genetic variability for each population are summarized in Table II. A total of 140 alleles with an average of 11.7 alleles per locus were observed across the 12 microsatellite loci, ranging from three (*SSOSL311*) to 23 (*Ogo2*). Across all populations, the average number of alleles per locus (A) was 4.0, the average mean allelic richness (A_R) was 3.2 and the average expected heterozygosity (H_E) was 0.42. European grayling populations, historically more strongly influenced by human caused stocking activities, displayed a significant higher ($P < 0.05$; two-sided test of the null hypothesis of no difference) level of genetic diversity (average $A_R = 3.7$ and $H_E = 0.46$) than populations without documented stock transfers (average $A_R = 2.8$ and $H_E = 0.36$). A total of 21 private alleles were found in 13 of 26 populations. The highest number of unique alleles (three) was found in populations Ege, Sin and InG. Allelic 'dropouts' in the data set would be expected to cause HW deviation due to excess of false homozygotes; however, no evidence of short allele dominance or large allele dropout was detected at any of the 12 polymorphic microsatellite loci analysed. Results based on the test by van Oosterhout *et al.* (2006) indicated potential presence of null alleles only in one population (Ilm) for the highly polymorphic loci *BFRO010* and *Ogo2* (with the combined probability of all allele size classes at each locus being not significant, $P > 0.05$). As there was no evidence of null alleles present in all other

TABLE II. Summary information on genetic diversity indices of the European grayling populations studied (see Table I for population codes)

Pop	n	No. hap.	No. lin.	mtDNA					Nucleo. div.	Hap. div.	n	A	AR	A _{pr}	H _E	H _O	P _{HW}	LD					
				Lineage frequency															Microsatellite loci				
				I	IIa	IIb	III	IV											A	A _R	A _{pr}	H _E	H _O
Ege	36	1	1	0.00	0.00	1.00	0.00	0.000	0.000	38	2.6	2.4	3	0.29	0.29	*	NS						
Tep	19	1	1	0.00	0.00	1.00	0.00	0.000	0.000	20	2.8	2.6	0	0.31	0.30	NS	NS						
Ssa	20	3	2	0.00	0.45	0.00	0.55	0.631	0.074	20	3.4	3.0	0	0.49	0.53	NS	*(1)						
Lam	13	3	2	0.00	0.54	0.00	0.46	0.579	0.072	13	3.1	2.8	1	0.45	0.52	NS	NS						
Vol	28	1	1	0.00	0.00	1.00	0.00	0.000	0.000	29	3.3	2.6	2	0.35	0.35	NS	NS						
Frs	15	2	2	0.00	0.66	0.33	0.00	0.460	0.043	15	2.8	2.7	0	0.45	0.42	NS	NS						
Sin	38	3	3	0.00	0.89	0.08	0.03	0.195	0.016	39	4.2	3.2	3	0.41	0.39	NS	*(1)						
Lei	30	3	3	0.00	0.90	0.03	0.07	0.188	0.018	30	3.5	2.8	1	0.26	0.22	***	** (2)						
WM	22	1	1	0.00	1.00	0.00	0.00	0.000	0.000	22	1.9	1.9	0	0.26	0.25	NS	NS						
Erf	15	2	2	0.00	0.87	0.00	0.13	0.239	0.018	15	4.1	3.7	0	0.51	0.46	*	NS						
BoS	38	2	1	0.00	1.00	0.00	0.00	0.366	0.009	39	2.4	2.2	0	0.23	0.21	NS	NS						
LiB	20	5	2	0.00	0.80	0.00	0.20	0.656	0.049	19	4.6	4.1	1	0.51	0.55	NS	NS						
InG	50	5	2	0.00	0.04	0.00	0.96	0.621	0.011	49	5.8	3.5	3	0.42	0.40	*	NS						
InA	23	4	2	0.00	0.09	0.00	0.91	0.244	0.012	23	4.8	3.6	0	0.49	0.55	***	*** (13)						
Saa	30	7	4	0.10	0.32	0.02	0.84	0.707	0.057	30	6.3	4.4	2	0.53	0.47	***	*** (22)						
TiA	37	9	3	0.14	0.27	0.00	0.59	0.855	0.064	39	6.1	4.3	1	0.54	0.54	*	*** (8)						
Am	36	5	2	0.00	0.03	0.00	0.97	0.631	0.012	34	4.2	3.3	0	0.42	0.37	***	*** (2)						
Isa	33	4	2	0.00	0.85	0.00	0.15	0.306	0.031	33	3.8	3.1	0	0.42	0.45	NS	*(1)						
Sem	38	6	2	0.00	0.05	0.00	0.95	0.657	0.026	39	4.1	3.4	0	0.49	0.48	NS	NS						
Ram	39	3	2	0.00	0.03	0.00	0.97	0.498	0.011	39	4.0	3.2	1	0.35	0.34	NS	*** (1)						
Ile	34	5	2	0.00	0.21	0.00	0.79	0.540	0.038	33	4.4	3.4	1	0.36	0.34	*	*** (9)						
Lec	18	4	2	0.00	0.17	0.00	0.83	0.686	0.026	20	4.8	3.7	0	0.43	0.43	NS	NS						
Fin	25	4	2	0.00	0.56	0.00	0.44	0.621	0.051	25	3.3	2.5	1	0.36	0.33	NS	NS						
WaN	10	4	2	0.00	0.50	0.00	0.50	0.695	0.062	10	2.4	2.7	0	0.43	0.51	NS	NS						
ScR	30	6	3	0.03	0.00	0.40	0.57	0.755	0.119	32	6.9	4.5	2	0.59	0.58	*	*** (2)						
Ilm	25	4	2	0.00	0.52	0.00	0.48	0.702	0.064	25	4.3	3.5	0	0.52	0.46	*	*** (2)						

MtDNA: n, sample size; No. hap., number of haplotypes; No. lin., number of lineages; frequency of lineages; Hap. div., haplotype diversity per population; Nucleo. div., nucleotide diversity per population. Microsatellite loci: n, sample size; A, average number of alleles or loci; A_R, mean allelic richness per population; A_{pr}, number of private alleles; H_E, expected heterozygosity; H_O, observed heterozygosity; P_{HW}, deviations from Hardy-Weinberg equilibrium; LD, linkage disequilibrium (number of locus pairs involved in parentheses). Significance after application of Bonferroni corrections: *P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant.

populations investigated, it can be assumed that not technical problems but rather the within population genetic structure of samples was responsible for the observed deviations in HWE and linkage disequilibrium (LD). Significant deviations from the expected HW proportions were observed in 11 populations (deficit of heterozygotes) and probability tests of LD were significant for 12 of the 26 populations after applying sequential Bonferroni corrections (Table II). After removing first and second generation immigrants (as identified by BAYE-SASS) for populations Lei and Saa and through separate analysis of sub-groups sampled from different river stretches for Am and InA, respectively, highly significant values for deviations for both HWE and LD disappeared. A Wahlund effect (Ohta, 1982) due to presence of different cohorts (through stocking and other reasons such as sampling of different year classes) probably explains the deviations in the remaining populations such as Ilm, Ile, ScR, TiA or ScR.

MICROSATELLITE DIVERGENCE AND ADMIXTURE ANALYSIS

On the two-dimensional plot of microsatellite-based F_{ST} values, the populations with high frequency of haplotypes from different mtDNA lineages (WaN, FiN, Isa, LiB, Ilm, Lam, Ssa, Erf and ScR) and populations where stocking has occurred from different drainages (Lei, Isa, Ilm, ScR, Ssa, TiA, Sem and Saa) were located between the 'pure' Elbe, Danube and Rhine and Main populations, indicating their admixed status [Fig. 2(a)].

The log likelihood of the genotypic data, $L(K)$ and ΔK , as estimated by the programme STRUCTURE, have a clear tendency to plateau for $K > 3$ (Fig. 3). Thus, a value of $K = 3$ can be considered as the most appropriate model for the uppermost hierarchical structure of the data. This also fits well with the three major mtDNA lineages that are found in the studied area of the Danube, Rhine and Main, and Elbe drainages (Gum *et al.*, 2005). STRUCTURE analysis, when applied separately for each population, provided evidence for multiple gene pools present within eight sampling sites: Lei, TiA, Saa and Ilm consisted of three gene pools ($K = 3$) while Isa, InA, Ile and ScR consisted of two clusters ($K = 2$). As expected, individuals from populations that historically have been more strongly affected by stocking with fish from different origins (*e.g.* Isa, Ilm, TiA or Saa) did not form a homogenous gene pool but were unequally distributed over two or three clusters, respectively. On the other hand, individuals of typical contact zone populations such as FiN or Ssa were also subdivided over two clusters but shared about equal proportions of individual admixture coefficients between these clusters.

INDIVIDUAL IMMIGRANT ANCESTRIES

Analysis of individual ancestries identified a majority of individuals in most populations as being 'non-immigrants' (Fig. 4). Some populations, especially those defined as 'stocked' in Table I (*e.g.* Isa, Ilm, TiA or Saa), however, showed a substantially higher proportion of individuals with recent immigrant ancestry. In population Ilm, a high number of both introgressed individuals as

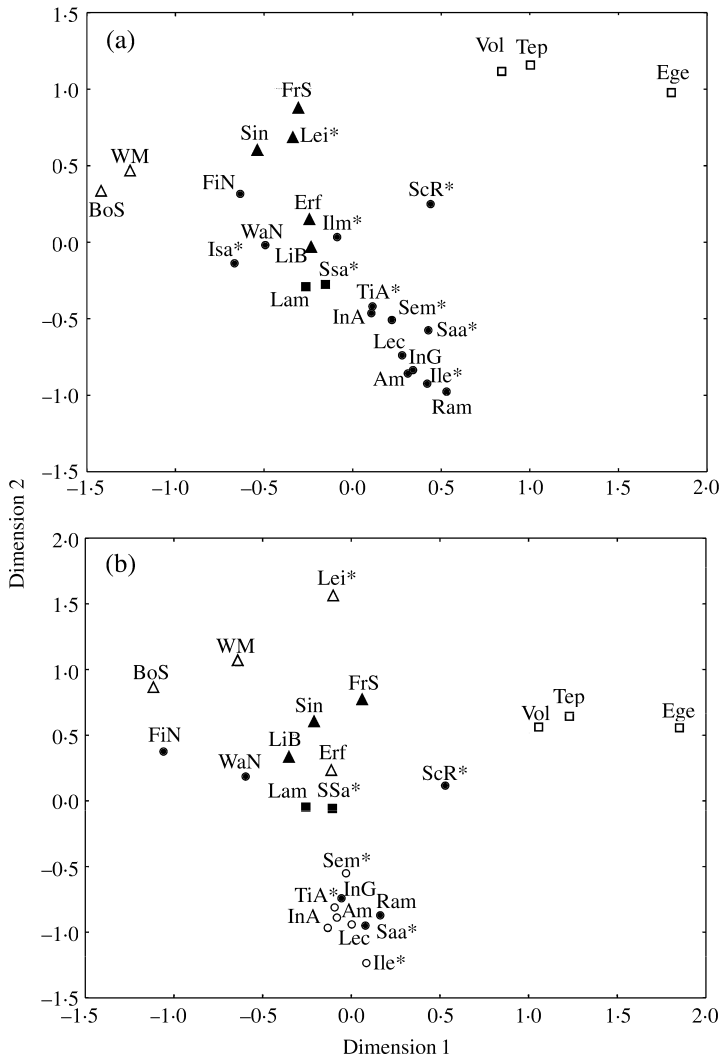


FIG. 2. Two-dimensional scaling analysis based on 12 microsatellite loci and pair-wise estimates of F_{ST} among populations (a) before ($n = 730$) and (b) after the exclusion of recent immigrants as identified by BAYESASS ($n = 532$): Danube (○, ●), Rhine and Main (△, ▲), and Elbe (□, ■). ●, ▲, ■, populations that possess different mtDNA lineages; * next to code, where stocking of European grayling originating from different drainages was documented at least once.

well as first generation immigrants were identified and only one of the 25 individuals remained non-immigrant with >90% posterior probability (Fig. 4). This result is certainly promoted by the fact of forced (*i.e.* human caused) mating of local and stocked fish that were performed at a local hatchery. A similar situation has to be proposed for populations TiA, Saa or Sem with the same hatchery involved to carry out the breeding programme. In population Isa, even all sampled European grayling were identified as first and second generation immigrants (Fig. 4). In populations Lei, Ile and Saa, several individuals

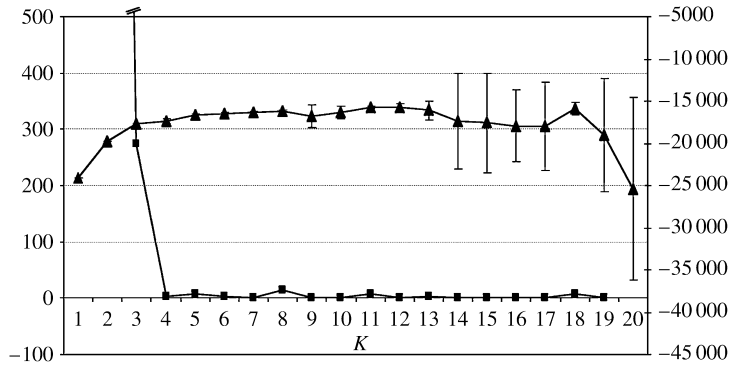


FIG. 3. Inference of genetic clusters (K) of the European grayling populations based on the STRUCTURE algorithm. Mean \pm s.d. of log-likelihood values (10 independent runs), $L(K)$ (▲), and the rate of change in the log probability of data between successive values of K , ΔK (■) for the whole data set ($n = 730$). The ΔK value for $K = 2$ ($= 3258$) is not shown.

(three, eight and 11, respectively) were identified as first or second generation immigrants and this was in agreement with the STRUCTURE analysis, which assigned the same individuals to another cluster with high confidence. In addition, based on mtDNA data, all of these individuals belonged to different mtDNA lineages compared to the majority of individuals sampled from these locations.

DISCRIMINATION OF HISTORICAL SECONDARY CONTACT FROM RECENT HUMAN MEDIATED STOCK TRANSFER

After removing the individuals with recent immigrant ancestries identified under the Bayesian assumption using microsatellite data, the proportions of different mtDNA lineages in the studied populations (except Isa where all individuals were identified as recent immigrants) were re-evaluated [Fig. 5(a)]. Substantial changes in the mtDNA lineage composition mainly occurred in

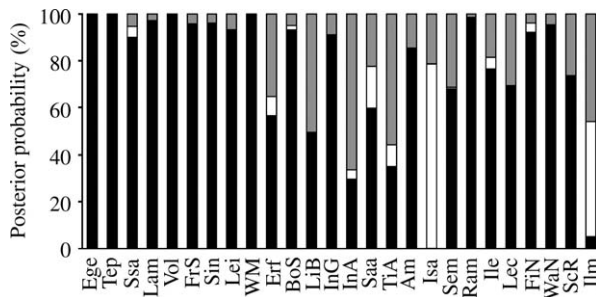


FIG. 4. Histogram of posterior probabilities of individual immigrant ancestries summarized for all European grayling populations as determined by BAYESASS 1.3 (population grouped by sub-drainage system). Proportion of individuals assigned to its source population are denoted 'non-immigrant' (■), proportion of an immigrant from a specific population denoted '1st immigrant' (□) and proportion of an offspring of an immigrant and a non-immigrant denoted '2nd immigrant' (▒).

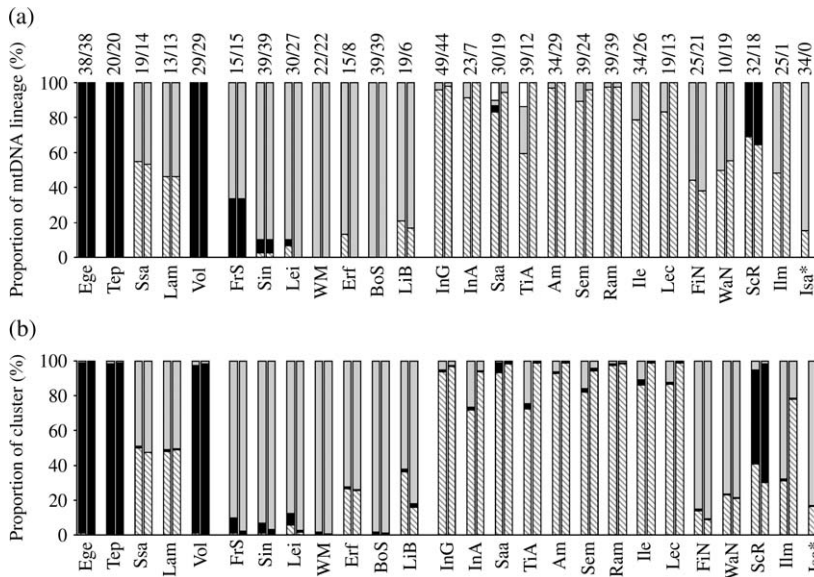


FIG. 5. (a) Proportions of major mtDNA lineages I (□), IIa (▨), IIb (■) and III (▤) within European grayling populations from Bavaria, and (b) estimates of population admixture coefficients [clusters 1 (□), 2 (■) and 3 (▨)] using STRUCTURE ($K = 3$). Shown are the proportions before (left bar) and after (right bar) the exclusion of recent immigrants and offspring among local and immigrant fish as identified by BAYESASS (see Fig. 2). (a) The sample size before and after exclusion of individuals is given above each bar. *Only the left bar for population Isa is shown because all individuals were identified as immigrants or introgressed with $>90\%$ probability and consequently excluded.

Danubian populations, which have been more strongly affected by stocking activities (TiA, Ilm, Saa and Ile), but also in some populations where only supportive breeding was known [InA, Lec, Am and the Main population Lei; Fig. 5(a)]. With exception of the Erf, the proportion of different mtDNA lineages changed only little in the other populations. Therefore, the high level of admixture of mtDNA lineages, which was retained in populations originating from contact zones of the drainage systems (such as FiN or Ssa) can be most probably explained by historical secondary contact. A substantially lower proportion of admixture was retained in populations from other sampling sites designated in Table I as ‘wild’ (e.g. Sin, InG and Ram). Though a substantial number of first and second generation immigrants were removed from populations LiB and ScR (12 and 14, respectively) only small changes in mtDNA lineage proportions were observed. Considering that ScR has been affected by stocking and supportive breeding, this suggests that the population obviously possessed divergent mtDNA lineages already prior to the recent human mediated transplantations.

A comparable result was obtained for microsatellite data as indicated by the estimates of population admixture coefficients using STRUCTURE [Fig. 5(b)]. If partitioned into $K = 3$ clusters according to the three drainages systems, again several populations showed clear changes in their cluster composition after removing of individuals with recent immigrant ancestries. Concerning

pair-wise estimates of F_{ST} based on the purged data set, the southern Danubian populations more clearly formed a separate group than before (Fig. 2(b)). In addition, samples Lei and FiN (three and four individuals removed, respectively) obtained a different position compared to Fig. 2(a), while no considerable change was observed for the remaining populations.

DISCUSSION

In this study, the combined analysis of nuclear and mtDNA data allowed the posterior proportions of divergent mtDNA lineages within European grayling populations under different fisheries management regimes to be estimated, *i.e.* to assess the level of historical secondary contact by excluding recent immigrants and introgressed individuals from phylogeographical analysis. In agreement with studies on related species such as the brown trout, *Salmo trutta* L., the present work showed that mtDNA data alone can only indirectly serve as an indicator of introgression (Weiss *et al.*, 2001; Duftner *et al.*, 2005), and do not allow discrimination between the historical and contemporary gene flow between the populations. Analysis of highly variable nuclear markers in conjunction with new statistical developments, however, has proved to be a valuable tool for identifying recent immigrants (Hansen *et al.*, 2001b). Most significantly, it has improved the possibilities for analysing admixtures between stocked and indigenous fishes at the species, population and individual levels (Pimmer *et al.*, 2000; Sušnik *et al.*, 2004; Hänfling *et al.*, 2005). Admixture analysis such as the STRUCTURE approach (Pritchard *et al.*, 2000) and the analysis of posterior probabilities of individual immigrant ancestries (BAYESASS; Wilson & Rannala, 2003), however, is dependent on the following conditions (Choisy *et al.*, 2004): (1) a high degree of differentiation between populations (high F_{ST} values), (2) a high ratio of native to stocked fish within populations and (3) a recent hybridization event accomplished with a well-documented management (stocking) history. Simulation analyses further showed that a larger number of loci (20) reduce bias and the mean square error, and that the posterior probability of each individual immigrant's ancestry can only be estimated with high confidence over the last few generations (Wilson & Rannala, 2003). For the European grayling populations studied, the above proposed necessary premises generally are fulfilled: first, a high level of differentiation is common for European grayling in central Europe (average $F_{ST} = 0.36$; Gum *et al.*, 2005) even between hydrologically connected sampling sites (Koskinen *et al.*, 2002; Gum *et al.*, 2003); second, natural migration of populations among geographically adjacent tributaries has been virtually impossible during the last century due to human caused habitat fragmentation (in Bavaria alone *c.* 9000 migration barriers such as hydropower stations or dams), and third, enhancement motivated stocking and supportive breeding has been carried out more intensively only for the last two decades.

An important constraint of the applied Bayesian method to discriminate between the historical secondary contact and recent stocking events certainly is the fact that individual immigrant ancestries can only reliably be estimated over the last several generations (Wilson & Rannala, 2003). The present approach, however, is still applicable and valid for the populations where

removing of recent immigrants results in the non-admixed sample, as estimated by the proportion of different mtDNA lineages [*e.g.* populations Ile, Saa, Lei, Lec in the data set; see Fig. 5(a)]. Of course, without the availability of historical baseline samples (*e.g.* scale samples; Nielsen *et al.*, 1997; Koskinen *et al.*, 2002) or knowledge of the genetic composition of stocked fish (Ruzzante *et al.*, 2004; Sušnik *et al.*, 2004) safe inferences about the genetic structure of populations can only be extended to few generations ago and they critically depend on the known stocking history. Keeping these limitations in mind the present study on European grayling showed that the recent advances in data analysis, such as Bayesian inference (Beaumont & Rannala, 2004) offer great opportunities for a better understanding of the effects of human activities on the genetic structure of wild populations.

GENETIC IMPACT OF STOCKING ON GENETIC STRUCTURE OF EUROPEAN GRAYLING

As inferred from the admixture analysis and the posterior probabilities of individual immigrant ancestries, the studied Bavarian European grayling populations fall into several overlapping categories: 'pure' populations (*e.g.* Sin, WM, Ege and Ram), several populations with a minor proportion of individuals with recent immigrant ancestries (*e.g.* Ssa, Lei and FiN), and several populations exhibiting relatively high levels of introgression (*e.g.* Isa, Ilm, InA and TiA). Even if natural interbreeding would play a minor role (possibly due to different spawning times or selection against hybrid offspring), admixture of exogenous and indigenous gene pools will occur as a result of forced mating through artificial reproduction. In the present study there was a good accordance between the results of genetic analysis and stocking records. For example, it is known that exogenous material of different origin has been introduced into the populations of Ilm, TiA or Saa and a major proportion of spawners from these small river stretches are caught and transferred to a hatchery each year for artificial reproduction. The results of genetic analysis confirmed this by revealing that the vast majority of individuals in these populations were either recent immigrants or introgressed. On the other hand, the study also showed that Bavarian European grayling populations, which have been enhanced by supportive breeding of local stocks (*e.g.* InG and Ram) and some exogenous material (*e.g.* ScR and Saa), have resisted the complete erosion of native genes although the documented stocking procedures could be traced genetically. In Finland, Koskinen *et al.* (2002) studied the genetic and ecological impact of stocking on European grayling populations, and similar to the report by Hanfland (2002), the stocked individuals exhibited poor survival and reproductive success compared to indigenous European grayling. In contrast, Sušnik *et al.* (2004) showed that ongoing stocking of European grayling originating from the Danube can lead to a loss of at least 50% of the native Adriatic type of European grayling in Slovenia. This observation is in line with the extent of introgression found for some southern Danubian populations of the present study. The unpredictability of the genetic impact of salmonid stocking activities was already pointed out by Hindar *et al.* (1991). The massive introduction of stocking exogenous genetic material into

autochthonous gene pools can result in (at one extreme) the displacement of indigenous stocks, negative consequences on the genetic structure of local fishes due to interbreeding with cultured fishes, increased hybridization rates with closely related species (Gross *et al.*, 2004), or (at the opposite extreme) no detectable introgression into indigenous gene pools despite substantial introductions (Hindar *et al.*, 1991). Moreover, stock transfer often leads to competition and genetic effects on performance traits such as stock size. Indirect effects may implicate earlier spawning leading to little recruitment or cultured fishes being maladapted to the environment in which they were released and reduced juvenile survival. Among the direct effects is 'contamination', *i.e.* introductions of pathogens, for example, the threat of Atlantic salmon *Salmo salar* L. from Norway through the transfer of Baltic Atlantic salmon and its associated parasite, *Gyrodactylus salaris* (Bakke *et al.*, 1990).

Important factors that determine the level of impact are the number of stocked fishes relative to the native population size (immigration rate) and the selection against the introduced fishes. Fritzner *et al.*'s (2001) study on brown trout concluded that even heavily stocked brown trout populations may be affected substantially less by hatchery brown trout than anticipated and should not immediately be given low conservation priority. Similar observations by Hansen (2002) shows that even heavily stocked salmonid populations may contain surprisingly low proportions of their gene pool derived from domesticated fishes, as in this case strong selection pressure may act against the stocked domesticated fishes. Angling was also shown to be an important factor influencing the genetic structure of fish populations and their management. Mezzera & Largiader (2001) demonstrated that 'selective angling, in combination with a small legal catch size may have considerably eliminated introduced brown trout and hybrids before spawning at the study sites, and thus may have reduced the introgression of alien genes into the local gene pool'. On the other hand, Fleming *et al.* (2000) investigated the lifetime success and interactions of farmed Atlantic salmon invading a native population and their results indicated that such annual invasions have the potential for impacting population productivity, disrupting local adaptations and reducing the genetic diversity of wild Atlantic salmon populations.

To sum up, a general rule for success and sustainability of stocking programmes is hard to find, but supportive breeding seems most promising for enhancement and conservation purposes (Wang & Ryman, 2001).

CONSERVATION PRIORITIES

In general, the European grayling populations studied in Bavaria serve as a good argument for the necessity of conducting genetic screening of stocked fish populations before making decisions about their conservation priority and status. It cannot be assumed *a priori* that offspring of stocked populations descend solely from hatchery fish and even heavily stocked populations may still contain indigenous genetic material of value for future conservation and restoration efforts. It would, therefore, be poor management to completely exclude some areas or rivers from conservation efforts and management, without determining whether the introgression is natural or anthropogenic and to what extent the native gene pool is still existing (Allendorf *et al.*, 2001).

As outlined above, for economical and ecological reasons, the stocking of cultured (domesticated) fishes is consistently considered detrimental when self-reproducing, indigenous populations are still present (Hindar *et al.*, 1991; Allendorf *et al.*, 2001). It must be stressed, however, that there is a large difference between domesticated strains and offspring from spawners caught in the wild and stocked back to their natal rivers (Lynch & O'Hely, 2001). Domesticated strains as typically produced for rainbow trout *Oncorhynchus mykiss* (Walbaum) and Atlantic salmon are hatched over several generations in artificial environments. Specifically, these individuals often exhibit unnatural behaviours, especially concerning their hiding response and antipredator behaviour (Hindar *et al.*, 1991; Cross, 1998). No comparable form of domestication has been achieved for European grayling in Bavaria yet, despite considerable efforts to create European grayling broodstocks in a few hatcheries (M. Hermann, pers. comm.). Fortunately, the remaining native gene pools of European grayling are currently not threatened by potential extensive releases of offspring from electrofished local or non-local European grayling as many local fishing authorities have actually stopped stocking European grayling due to the ongoing cormorant and goosander debate (Cowx, 2005; Behrens *et al.*, in press). Until this problem is solved, the establishment of captive breeding programmes and more intensive supportive breeding actions will remain at a relatively low level (Bavarian Fishing Association, pers. comm.). This reprieve, however, is being challenged by the advocacy by regional fishing authorities and private angling associations for local breeding programmes and the use of offspring from locally caught spawners for future enhancement. In this respect the data of this work can be used for the identification of non-introgressed individuals or populations that could be used for supportive breeding.

Given the increasing efforts to establish conservation units for European grayling, the genetic discrimination between natural and human caused admixture is of particular interest and critical value in the contact zones of the Danube, Main and Elbe drainages. As has been shown in the FiN and Ssa populations, *c.* 50% proportion of two highly divergent mtDNA lineages within these populations remained constant after the exclusion of recent immigrants. Therefore, it can be concluded that these zones have been long-standing areas of secondary contact among these lineages over an extended period of time due to glacial perturbations. The area of the lower mountain range of the 'Fichtelgebirge', obviously represents a 'hot spot' of aquatic biodiversity in central Europe. Several evolutionary significant units or management units of both vertebrate (Hänfling & Brandl, 1998; Englbrecht *et al.*, 2000; Gum *et al.*, 2005) and invertebrate species such as the critically endangered pearl mussel *Margaritifera margaritifera* L. intersect here (Geist & Kuehn, 2005). The general outcome of these studies corroborates the need to change the concept of 'single species protection' towards conservation of ecosystems. In addition, further studies within the framework of functional genomics (Ranz & Machado, 2006) are needed in the studied region to clarify the level of local adaptation of specific units to different environmental conditions.

Based on multilocus genotypic data this study showed that it is possible to discriminate long-term historical gene flow (natural secondary contact) from

recent immigration and introgression over the last few generations. Through the application of the latest computational achievements (Wilson & Rannala, 2003) Bayesian inference allowed to identify immigrant and introgressed individuals within European grayling populations that were affected by human stocking activities. In combination with small- and large-scale phylogeographic information of the studied area the present work exemplified a way to separate historical and natural from human caused admixture of divergent mtDNA lineages. This approach could therefore also be applied to other species influenced by human introductions. The question of whether different mtDNA lineages originate from 'man or nature' can adequately be answered, provided that the system under study still consists of a mixture of the founding sub-groups where panmixia has not yet been reached.

Thus, the presently available molecular techniques and statistical methods allow for a comprehensive genetic analysis of wild populations, without requiring an exhaustive knowledge of the management history. It is possible to provide *ad hoc* information on important variables such as the number of sub-groups present within populations as well as to determine the level of interbreeding and historical gene flow. In this regard the results provide the basis for future management and conservation efforts of European grayling in Bavaria.

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