Conservation Genetics **4:** 735–747, 2003.

Fragmentation by weirs in a riverine system: A study of genetic variation in time and space among populations of European grayling (*Thymallus thymallus*) in a Danish river system

Torben Meldgaard[1,2,3], Einar E. Nielsen[2,*] & Volker Loeschcke[1]

[1]*Department of Genetics and Ecology, University of Aarhus, Building 540, Ny Munkegade, DK-8000 Aarhus C, Denmark;* [2]*Danish Institute for Fisheries Research, Department of Inland Fisheries, Vejlsøvej 39, DK-8600 Silkeborg, Denmark;* [3]*Currently "Ecosystèmes Lagunaires", Equipe EFEP, Université Montpellier II, CC 093. Place E. Bataillon, 34095 Montpellier, Cedex 5, France (*Author for correspondence: Phone: +45 89 213144; Fax: +45 89 213150; E-mail: een@dfu.min.dk)*

Received 28 February 2002; accepted 31 October 2002

Key words: ancient DNA, assignment tests, fragmentation, microsatellite DNA, temporal variation, *Thymallus thymallus*, weirs

Abstract

Human induced habitat alterations affect the genetic structure of many fish populations. Weirs in particular have caused fragmented populations previously connected by gene-flow. We studied the effects of weirs on the distribution of genetic variation within European grayling (*Thymallus thymallus*) populations from the Skjern River, Denmark. We compared microsatellite data from DNA extracted from historic scale samples collected 60 years ago with data from contemporary samples.

Pairwise multilocus F_{ST} estimates between all contemporary population samples were significant as well as exact tests for population differentiation. Assignment tests of individuals to a set of baseline samples showed correct assignment to the population of origin of between 54 and 79%. Assignment of individuals from recent samples to the historic population samples showed highly variable results (3–83%) of correct assignment suggesting different population histories. Pairwise multilocus F_{ST} estimates were significantly correlated with the number of intervening weirs but not with waterway distance. A simulation procedure was used to estimate differences in relative population sizes, which indicated that the main river population was approximately three times larger than those of the tributaries. There were no signs of any loss of genetic variation for the river system as a whole during these 60 years. The results show that weirs can be an important factor for creating the current distribution patterns of genetic variation among grayling populations, most likely by only allowing passive downstream drift of fry and obstructing active upstream migration.

Introduction

Fish populations all over the planet have experienced an increase in human induced habitat alterations affecting their living conditions. The building of weirs has caused fragmentation (Northcote 1995); pollution, overfishing and degradation of habitats have in some cases led to the loss of entire populations. In other cases, populations have been able to adapt to these environmental changes. For populations to adapt, the presence of sufficient genetic variability is a prerequisite. However, fragmentation of populations can lead to an increase in genetic drift which eventually leads to loss of rare alleles, thus reducing total genetic variability (Hedrick and Miller 1992). In extreme cases, fragmentation can lead to inbreeding and inbreeding-depression, which have been shown to reduce the fitness of fish species (Vriejenhoek 1994).

Although commercially not as important as other salmonid species such as Atlantic salmon (*Salmo*

salar) and trout (*S. trutta*), grayling (*Thymallus* spp.) is considered a culturally important species, particularly for recreational fishing. Factors such as alterations of water quality and overfishing have induced several declines in local population size of grayling in many parts of Europe and North America (Northcote 1995). These declines may have been the main factor leading to the increased interest in genetic research concerning grayling (Redenbach and Taylor 1999; Sušnik et al. 1999a; Sušnik et al. 1999b; Sušnik et al. 2000; Koskinen and Primmer 1999; Koskinen et al. 2000; Koskinen et al. 2002). However, to our knowledge, no work has been performed concerning the genetic consequences of fragmentation by weirs, although it is known that grayling do not use traditional bypasses to a satisfactory extent (Jungwirth 1994 and references therein).

The analysis of microsatellites has proven to be a powerful tool in the quest for further knowledge about the genetic structure of salmonid fishes, especially when microgeographic relationships are the subject of interest (Estoup et al. 1998; Carvalho and Hauser 1998; Hansen et al. 1999). The power of microsatellite analysis relates to features such as high levels of polymorphism and amplification of poor quality and low quantity of DNA, which permits analyses of DNA from old scale samples (e.g. Purcell et al. 1996, Miller and Kapuscinski 1997; Nielsen et al. 1997; Nielsen et al. 1999a; Nielsen et al. 1999b; Tessier and Bernatchez 1999). Until recently, only few microsatellite loci derived from grayling were known, but due to the possibility of cross-species amplification, an increased number of polymorphic loci are now available (Koskinen and Primmer 1999), which increases the statistical power of genetic analysis.

In this paper we present the results of a study on grayling from the Skjern River system in Denmark. The populations within the river system have decreased considerably in size within the past 50 years (Ernst and Nielsen 1981). In the same period, many fish farms were built. To facilitate fish migration and thereby prevent fragmentation of the populations, traditional fish ladders were built. Studies have shown that the traditional types, although adequate for adult brown trout (*Salmo trutta*) passage, constitute insurmountable migration obstacles for grayling (Jungwirth 1996 and references therein). We investigated whether 60 years of fragmentation and occasional pollution led to changes in the genetic composition of the populations and loss of genetic variation. To obtain genetic data prior to any severe population size declines and fragmentation, we extracted DNA from scale samples collected in the 1940s. We compared the genetic composition of the populations 60 years ago with genetic data from contemporary samples in order to determine the genetic relationship between the Skjern River populations of the 1940s and the present populations, and to evaluate possible changes in the levels of genetic variability. We also compared the distribution of genetic variation among present populations and applied a novel Markov Chain Monte Carlo simulation approach, based on the observed distribution of microsatellite alleles and their repeat numbers (Beaumont 1999) to gain information about the possible occurrence and magnitude of declines in population sizes. This approach was also applied to assess the relative difference in population sizes.

Table 1. Year of construction and year of new bypass for the eight weirs separating sample sites in the Skjern River system

Number	Name	Year of construction	Year of new bypass
1	Nr. Vium fish farm	1953	1992
2	Kideris fish farm	1964	1992
3	Høgild fish farm	1953	1991
4	Skovbjerg eel trap	<1888	1989
5	Hyttens fish farm	1966	1992
6	Flø fish farm	1942	1987
7	Harrildgaard fishery	1950	1997
8	Harrild water mill	<1940	2001

Materials and methods

Sampled populations

The Skjern River system is the largest river system in Denmark, draining approximately 2500 km^2 of land. Numerous fish farms, with associated weirs to ensure sufficient intake of water, were constructed during the 1940s, 50s and 60s (Table 1). Bypasses were build to avoid fragmentation, however, the traditional form of bypass was a "pool and weir"-type in which the fish had to jump or swim from chamber to chamber. Only recently (1987–present) these have been replaced with the "state-of-the art" bypass, which simply consists of a small bypass stream.

Scales from grayling from the Skjern River (SKJ40) and from two tributaries, the Vorgod River (VOR40) and the Holtum River (HOL40) were sampled from 1940 to 1946 by anglers and stored in

small paper bags. The exact location of sampling sites was not available for most individuals. Electrofishing was carried out in the Skjern River in 1997 (SKJ97) as well as in its tributaries the Holtum River (HOL00), the Fjederholt River (FJE00) and the Vorgod River (VOR00) in August and September 2000. Tissue samples from the adipose fin were taken and stored in ethanol.

The Fjederholt River had, according to local anglers, experienced a decrease in population size within the last decade, and was therefore included in the modern survey. It drains approximately 130 km^2 of land (mean annual discharge approximately 1.5 m^3 s^{-1}) and is characterised by long stretches of sandy bottom and low water velocity with low density of grayling. In the Holtum River, which drains approximately 210 km^2 of land (mean annual discharge approximately 2.7 m^3 s^{-1}), no grayling were observed upstream the weir at Harrild water mill (Figure 1) in our study, as opposed to observations from Ernst and Nielsen (1981), although long stretches seemed very suitable with high water velocities and gravel at the bottom. The Vorgod River, which is the largest of the studied tributaries (draining 450 km^2, mean annual discharge approximately 6.8 m^3 s^{-1}), seemed to have high densities of grayling. The river has a history of severe pollution from fish farms, dairies, farms and sewage treatment works as well as discharges of ochre and acidic water from the mining of brown coal. The weir at Nr. Vium fish farm splits the VOR00 samples and the weir at Harrildgaard fish farm splits the HOL00 samples.

As suggested by Ruzzante (1998) sample size was set to 50 individuals of various age-classes, except for the Skjern River population in which only 35 individuals had been sampled in 1997. In the historic populations the numbers of individuals varied considerably, ranging from 17 in the Vorgod River to >35 in the Holtum and Skjern rivers.

Microsatellite DNA analysis

DNA was successfully extracted and amplified from more than 90% of the old scale samples. Extraction procedures followed Nielsen et al. (1999b). For recent samples (tissue stored in ethanol) we employed the method of Estoup et al. (1996) for DNA extraction. Eight dinuclotide microsatellite loci were assayed: One2 (Scribner et al. 1996), Ogo2 (Olsen et al. 1998), OmyFgt1TUF (Sakamoto et al. 1994), SsBglIIM.26 (Goodier, unpublished, GenBank Accession no. U10051), BFRO011 (Sušnik et al. 2000), and BFRO015, BFRO017 and BFRO018 (Sušnik et al. 1999b). Since we wanted to amplify DNA extracted from old scales, the length of the amplification products was taken into consideration as we avoided loci with lengths of more than 300 bp. The following three loci were screened but left out because of low polymorphism: BFRO005, BFRO007 (Sušnik et al. 1999a) and BFRO010 (Sušnik et al. 2000). The locus BFRO008 (Sušnik et al. 1999a) seemed to have a high degree of variation but did not produce scorable alleles due to high levels of stutter bands.

The PCR annealing temperature was 54 °C for One2, Ogo2, OmyFgt1TUF, BFRO011, BFRO015, BFRO017 and BFRO018. For SsBglIIM.26 the annealing temperature was 52 °C. One of two different cycling profiles was applied: (*i*) applied to: BFRO011, BFRO015, BFRO017 and BFRO018: pre-denaturation at 95 °C for 5 min. followed by 30 (fresh samples) or 40 (scale samples) cycles with denaturation at 95 °C for 30 s, annealing at 52–54 °C for 30 s, extension at 72 °C for 30 sec. and a final extension at 72 °C for 5 min; (*ii*) applied to: One2, Ogo2, OmyFgt1TUF and SsBglIIM.26: a touchdown PCR profile where the annealing temperature was decreased 1 °C every 6th cycle (54–48 °C). The difference in number of PCR-cycles, between old and fresh samples, was applied in order to increase yield. One of each primer was end-labelled with the fluorescent dye CY5, and the microsatellites were analysed on a Pharmacia ALFexpress automated sequencer (Pharmacia, Uppsala, Sweden) according to the manufacturer's recommendations. Gels were scored using ALLELINKS version 1.00 (Pharmacia). In order to verify the reproducibility of results, 27 (9 from each river) individuals from the historic samples as well as 24 (6 from each river) individuals from contemporary samples were genotyped twice at each locus. No differences in scored genotype between runs were observed. Potential problems of large allele dropout (higher degree of amplification of the shorter allele) were minimised by overloading the ALFexpress with PCR-product from presumed homozygotes. Furthermore, as recommended by Nielsen et al. (1999b), we used negative controls (PCR reactions where no template was been added) to check for cross-and/or aerosol contamination.

Statistical treatment

Only individuals for which all eight loci were successfully scored were included in the statistical analysis.

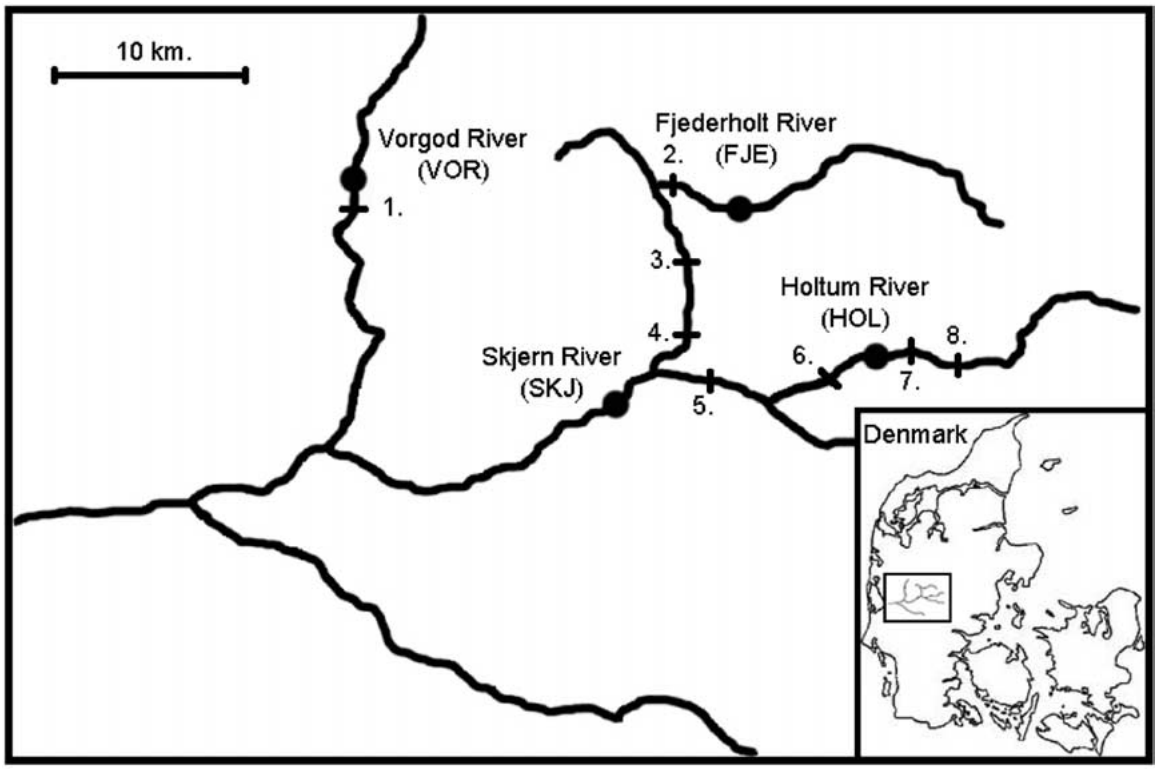

Figure 1. The Skjern River system. Black dots indicate the centres of sampling sites. Abbreviations are shown in parentheses. Numbers indicate weirs: (1) Nr. Vium fish farm, (2) Kideris fish farm, (3) Høgild fish farm, (4) Skovbjerg eel trap, (5) Hyttens fish farm, (6) Flø fish farm, (7) Harrildgaard fish farm, (8) Harrild water mill.

Departure from Hardy–Weinberg equilibrium and linkage disequilibrium between pairs of loci were tested by an exact test (Guo and Thompson 1992) using an updated version (3.2a) of the GENEPOP package (Raymond and Rousset 1995a). Observed and expected heterozygosities of the markers were estimated using the program POP100GENE (Piry and Bouget 1999). Genetic differentiation between populations was estimated using pairwise F_{ST}-estimates and their significances were tested according to Weir and Cockerham (1984), with the program FSTAT (Goudet 2000). Because of the limited geographical and temporal scale of this study we chose to use F_{ST} rather than R_{ST} as a measure of genetic differentiation (Slatkin 1995; Rousset 1996). Exact tests for differences in allele frequencies (Raymond and Rousset 1995b) were performed and single locus probabilities were combined over loci by Fisher's method. The levels of significance for multiple tests were adjusted by the sequential Bonferroni method (Rice 1989). Isolation-by-distance i.e. the correlation between matrices of pairwise multilocus F_{ST} estimates and waterway distances (geographical distances measured following river contours using the Geographical Information System (GIS)) were tested using the Mantel permutation procedure (10.000 permutations) with the Spearman rank correlation coefficient as test statistic (Mantel 1967). The same procedure was used to test for correlation between pairwise multilocus F_{ST} estimates and number of intervening weirs between samples (isolation-by-weirs). Despite numerous tributaries within the river system, we considered it to be a one-dimensional habitat, which therefore allowed us to analyse isolation-by-distance by regressing multilocus pairwise $F_{ST}/(1 - F_{ST})$ estimates over waterway distance for all pairs of contemporary populations (Rousset 1997).

To get further information about the distinctness of populations, we performed assignment tests (Paetkau et al. 1995) using the program GENECLASS 1.00.02 (Cornuet et al. 1999) and the included "Bayesian approach" (Rannala and Mountain 1997). Briefly, for each individual the marginal probability in each

population was calculated, based on the allele frequencies of the samples, and an individual was assigned to the sample in which it had the highest marginal probability. A potential problem with this procedure is that all individuals are assigned to one of the samples even if they are derived from a different, unsampled population. The Geneclass program contains a 'simulation' option for assessing the probability of a multilocus genotype being derived from the allele frequencies of a population. A number of multilocus genotypes (in this case 10,000) were randomly generated, based on the estimates of allele frequencies of the specific population, and a frequency distribution of marginal probability values was generated. The marginal probability of a specific individual multilocus genotype was then compared to the distribution of marginal probabilities of randomly generated multilocus genotypes, and if the value was below a certain threshold probability level, the individual was 'rejected' from the sample. We performed self-classification runs for the populations from 1940 and 2000, and assigned the 2000 populations to the populations from 1940.

Finally, we used the procedure by Beaumont (1999) for detecting population declines and expansions. The applied program MSVAR assumes a strict Stepwise Mutation Model (SMM) and estimates the posterior probability distribution of several genealogical and demographic parameters. This is done using Markov Chain Monte Carlo simulations, based on the observed distribution of microsatellite alleles and their repeat numbers. The program produces the following three important parameters: r, defined as N_0/N_1 where N_0 is the current effective number of chromosomes, and N_1 is the number of chromosomes at the time where the expansion/decline began, t_f which is defined as t_a/N_0 where t_a denotes the number of generations since the beginning of the expansion/decline and finally θ, defined as $2N_0\mu$, where μ denotes the mutation rate. We performed the analyses for all seven populations, assuming both linear and exponential modes of decline/expansion. As proposed by Beaumont (1999), we ran the program on each population five times with different starting points of the chains in order to gain knowledge of the variability of estimates. The results should not be biased by systematic errors due to differences in sample size as sample size is taken into account in the calculations. However, with smaller samples less can be inferred about the demographic parameters (Mark Beaumont, personal communication). Furthermore, as described by Beaumont (1999), the results are likely to be biased by the sole use of polymorphic loci, which means that r and t_a will be overestimated to some degree.

Results

The total number of alleles per locus across the populations varied from four at the locus OmyFgt1-TUF to ten at the locus BFRO011 (Table 2). In individual population samples the number of alleles ranged from three to eight (Table 2). Eight private alleles (alleles which are exclusively found in one population) were found. Two of the private alleles were found in SKJ97 at the locus BFRO011. Another two were only found in the HOL populations while the remaining four all were found at different loci in different populations. None of the private alleles were found at frequencies above 5%. The level of expected heterozygosity for individual loci within populations varied between 0.28 (SsBglIIM.26, VOR40) and 0.82 (BFRO015, HOL40). Exact tests for linkage disequilibrium between loci gave 14 significant tests at the 5% level out of 196 pair-wise comparisons. However, no pairs of loci exhibited significant deviations from linkage equilibrium in more than one population and none of the individual tests proved significant following Bonferroni correction (data not shown). Significant deviations from Hardy-Weinberg proportions were found at the 5% level in 6 out of 56 probability tests, but none of them was significant after the Bonferroni correction (*P*-values not tabulated).

The least differentiation between historic and contemporary samples occurred between the SKJ and the HOL populations (F_{ST} = 0.014 and 0.020 respectively, Table 3). The largest pairwise F_{ST}-values were found between FJE and HOL (F_{ST} = 0.095) in 2000 and SKJ and VOR (F_{ST} = 0.058) in 1940. Genetic differences between temporal samples within rivers (Table 3) were also significant. The difference between the two SKJ samples was significant at the 5% level and the differences between temporal HOL and VOR samples at the 0.1% level. Estimates of pairwise F_{ST}-values ranged from 0.007 (SKJ) to 0.050 (VOR). Bootstrapping over loci (Table 3) revealed large and overlapping 95% confidence intervals, calling for careful handling of comparisons of pairwise F_{ST}-values. In the 1940 populations there were non-overlapping 95% confidence intervals of pairwise F_{ST}-values between SKJ-HOL and VOR-HOL, whereas in temporal comparisons this was only

Table 2. Summary of data on genetic variability in the sampled populations of European grayling in the Skjern River system. Shown are the number of alleles per locus (number of private alleles in parentheses, an asterisk denotes that the allele is found in both the 1940 and the 2000 populations), outcome of k = 56 tests for expected (H_e) and observed (H_o) heterozygosity and sample sizes (n). Deviations from expected Hardy-Weinberg proportions were all non-significant Table-wide significance levels were applied, using the sequential Bonferroni technique (Rice 1989)

		Population						
Locus	Statistic	FJE00	HOL00	VOR00	SKJ97	HOL40	SKJ40	VOR40
BFRO017	No. alleles	5 (1)	3	4	3	3	3	3
(total 5 alleles)	H_e	0.683	0.547	0.669	0.633	0.592	0.664	0.492
	H_o	0.667	0.478	0.75	0.714	0.552	0.679	0.538
	n	48	46	48	35	29	28	13
BFRO015	No. alleles	5	6 (1*)	6	5	6(1*)	6	5
(total 7 alleles)	H_e	0.729	0.745	0.791	0.735	0.817	0.813	0.748
	H_o	0.729	0.804	0.792	0.771	0.759	0.714	0.692
	n	48	46	48	35	29	28	13
One2	No. alleles	4	4	4	4	4	5	5
(total 5 alleles)	H_e	0.670	0.580	0.654	0.709	0.666	0.741	0.683
	H_o	0.729	0.522	0.688	0.657	0.552	0.607	0.692
	n	48	46	48	35	29	28	13
OmyFgt1TUF	No. alleles	3	3	3	3	4 (1)	3	3
(total 4 alleles)	H_e	0.557	0.665	0.655	0.661	0.633	0.658	0.615
	H_o	0.438	0.652	0.583	0.514	0.724	0.786	0.615
	n	48	46	48	35	29	28	13
BFRO018	No. alleles	5	6 (1*)	5	5	5 (1*)	5	4
(total 6 alleles)	H_e	0.742	0.746	0.745	0.757	0.679	0.736	0.566
	H_o	0.688	0.717	0.750	0.571	0.759	0.536	0.538
	n	48	46	48	35	29	28	13
Ogo2	No. alleles	5	5	6	6	5	5	5
(total 6 alleles)	H_e	0.673	0.544	0.754	0.623	0.635	0.611	0.548
	H_o	0.604	0.522	0.771	0.600	0.483	0.536	0.615
	n	48	46	48	35	29	28	13
SsBglIIM.26	No. alleles	3	4	6 (1)	5	4	5	3
(total 7 alleles)	H_e	0.499	0.351	0.591	0.581	0.378	0.518	0.280
	H_o	0.438	0.370	0.438	0.543	0.379	0.571	0.231
	n	48	46	48	35	29	28	13
BFRO011	No. alleles	6 (1)	6	6	8 (2)	5	6	6
(total 10 alleles)	H_e	0.390	0.777	0.602	0.782	0.721	0.695	0.745
	H_o	0.313	0.761	0.688	0.743	0.690	0.679	0.846
	n	48	46	48	35	29	28	13

the case between SKJ and VOR. Tests for population differentiation within the populations now connected by modern bypasses (VOR00 and HOL00) were not significant (data not shown). The Mantel tests revealed a significant positive correlation between the number of intervening weirs and pairwise F_{ST} estimates, as the probability of the association being random was 0.0455 (one-tailed test, Spearman rank correlation coefficient; Figure 2). We did not reveal a significant positive correlation between pairwise F_{ST} estimates and distance (one-tailed test, $P = 0.209$).

The assignment tests which involved samples taken in the year 2000 showed that a high proportion of individuals from the tributaries FJE00, HOL00 and VOR00 were assigned correctly, i.e., to the samples they were derived from (79%, 67% and 75% respectively), whereas a lower proportion (54%) were assigned correctly in the main river (SKJ97) (Table 4a). From HOL00 24% of the individuals were misassigned to SKJ97 and 29% from SKJ97 were misassigned to HOL00. In the 1940 populations (Table 4b) the correctly assigned proportions were 72%, 61% and 77% for HOL40, SKJ40 and VOR40 respectively. As in the contemporary populations, a considerable number of individuals (28%) from SKJ40 were assigned to HOL40 and vice versa (32%). We also assigned the contemporary population samples to the 1940 population samples (Table

Table 3. Tests for genetic differentiation between pairs of samples. Above diagonal: 95% confidence intervals for estimates of F_{ST} based on bootstrapping over loci. Below diagonal, first line: exact tests for differences in allele frequencies between samples, Second line: F_{ST}-values for pairwise comparisons of the populations sampled. Values in italics concern spatial comparisons only. Values in bold concern temporal differentiation within rivers. Table-wide significance levels were applied, using the sequential Bonferroni technique (Rice 1989) (k = 28)

	FJE00	HOL00	VOR00	SKJ97	HOL40	SKJ40	VOR40
FJE00	–	*0.038–0.156*	*0.023–0.147*	*0.015–0.145*	0.024–0.181	0.022–0.143	0.053–0.233
HOL00	***	–	*0.012–0.066*	*0.004–0.037*	**0.009–0.045**	0.010–0.057	0.024–0.097
	*0.095***						
VOR00	***	***	–	*0.006–0.058*	0.012–0.030	0.001–0.028	**0.023–0.073**
	*0.071***	*0.038***					
SKJ97	***	***	***	–	0.007–0.035	**−0.007–0.021**	0.021–0.094
	*0.073***	*0.020***	*0.030***				
HOL40	***	***	***	***	–	*0.007–0.021*	*0.031–0.094*
	0.097***	**0.027****	0.022***	0.021***			
SKJ40	***	***	*	**	**	–	*0.014–0.104*
	0.077***	0.031***	0.014*	**0.007***	*0.014***		
VOR40	***	***	***	***	***	***	–
	0.134***	0.066***	**0.050****	0.060***	*0.062****	*0.058****	

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

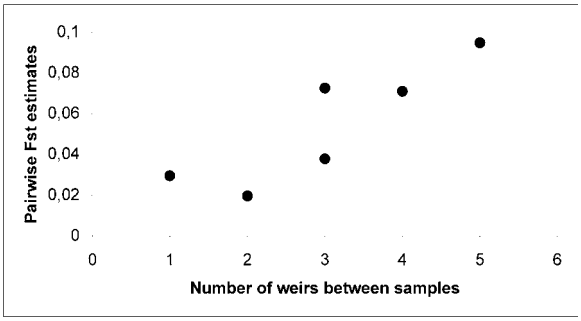

Figure 2. Plot showing the relationship between pairwise F_{ST} estimates and number of intervening weirs between samples.

4, section c). The proportion of contemporary individuals assigned to the old population varied considerably among samples. 83% of the SKJ97 individuals were assigned to SKJ40, 56% of the individuals from the HOL00 sample were assigned to HOL40, while only 3% of the VOR00 were assigned to the VOR40 sample.

The MSVAR program from Beaumont (1999) used for assessing population declines and expansions suggested that the population sizes in all populations had declined to approximately 25–33‰ of the original population size (r-values, Table 5). As we did not have a precise estimate of the current population sizes (N_0), it was not possible to estimate the time when the population decline began with satisfactory precision. However, assuming a generation time of three years and population sizes (N_0) of at least 100 individuals, the analysis suggested that this decline began at least 7500 years ago. We also used the program for estimating relative differences in the population sizes (N_0), as we assumed a fixed mutation rate (μ). This was done since the same set of microsatellite loci was used in all populations. Thus, $\theta/2$ was used as a relative measure of population size. The data from present time (Figure 3) suggested that SKJ97 ($\theta/2$ = 0.215) was approximately three times larger than the other populations. VOR00 and HOL00 ($\theta/2$ = 0.076 and 0.075 respectively) were of equal size, and FJE00 ($\theta/2$ = 0.061) was significantly smaller than HOL00, but not significantly smaller than VOR00 due to large standard deviations of the VOR00 estimate. For samples within rivers, SKJ97 was more than twice as large as SKJ40 ($\theta/2$ = 0.086). There was no significant difference between HOL00 and HOL40 and VOR40 ($\theta/2$ = 0.178) was approximately twice as large as VOR00. These results were based on a linear model of population decline. Simulations assuming an exponential model also pointed to a strong decline long ago, but as this model is primarily valid for short-term strong declines (Beaumont 1999), we emphasise the results based on a linear model.

Table 4. Results of assignment tests to estimate the most probable population of origin of individuals using the "Bayesian" approach of the Geneclass software by Cornuet et al. (1999). Each individual is *assigned* to the sample in which it has the highest likelihood of belonging. *Rejected* refers to the proportion of individuals from the sample of consideration that are rejected at the 0.05 level from the baseline samples. *n* refers to the number of individuals in each sample

Sample	Assistant	FJE00	HOL00	SKJ97	VOR00	Rejected from all samples
(a)						
FJE00	Assigned	0.79	0.13	0.04	0.04	
($n = 48$)	Rejected	0.13	0.69	0.33	0.23	0.06
HOL00	Assigned	0.04	0.67	0.24	0.04	
($n = 46$)	Rejected	0.70	0.13	0.13	0.20	0.02
SKJ97	Assigned	0.06	0.29	0.54	0.11	
($n = 48$)	Rejected	0.69	0.40	0.23	0.26	0.17
VOR00	Assigned	0.04	0.06	0.15	0.75	
($n = 35$)	rejected	0.73	0.50	0.38	0.17	0.13
(b)						
			HOL40	SKJ40	VOR40	
HOL40	Assigned		0.72	0.28	0.00	
($n = 29$)	Rejected		0.17	0.14	0.86	0.07
SKJ40	Assigned		0.32	0.61	0.07	
($n = 28$)	Rejected		0.46	0.11	0.89	0.07
VOR40	Assigned		0.08	0.15	0.77	
($n = 13$)	Rejected		0.62	0.08	0.31	0.00
(c)						
HOL00	Assigned		0.56	0.40	0.04	
($n = 46$)	Rejected		0	0.02	0.54	0
SKJ97	Assigned		0.10	0.83	0.07	
($n = 48$)	Rejected		0.31	0.06	0.54	0.06
VOR00	Assigned		0.37	0.60	0.03	
($n = 35$)	Rejected		0.14	0	0.63	0

Discussion

The present study revealed a significant isolation-by-weir effect among the contemporary populations. Obviously, the number of intervening weirs between sample sites is often likely to be positively correlated with distance. However, our analysis did not reveal a significant isolation-by-distance effect among populations, which is in accordance with most surveys on resident salmonids (Ryman 1983; Hansen and Loeschcke 1996; Carlsson et al. 1999). Hence, there are clear indications that weirs have affected the genetic structure of grayling in the studied river system. We consider the Skovbjerg eel trap in the Fjederholt River (Figure 1, n. 4) to be the main factor of the higher pairwise F_{ST}-values between the Fjederholt river samples and the others. For a period of at least hundred years (<1888–1989), the Skovbjerg eel trap was a practically impassable obstacle to the fish in the river as no bypass existed at all. The result is in concordance with earlier studies on resident salmonids in Scandinavia separated by impassable structures (Skaala and Nævdal 1989; Hindar et al. 1991a; Carlsson et al. 1999). Carlsson and Nilsson (2001) were able to show that differentiation of populations of resident brown trout was determined by a combination of distance, impassable waterfalls, high-velocity rapids and subdivision into tributaries.

Our analysis demonstrates that the genetic composition in the main river has not changed significantly over 60 years. In the tributaries, the Vorgod and Holtum rivers, however, there were signs of a genetic change in the same period. This is not surprising as small populations are prone to genetic drift to a larger extent than large populations. A large population will tend to be at mutation-drift equilibrium, which means that genetic variation lost by genetic drift is replaced by new alleles from mutation. As the effective population size decreases, random processes tend to change the allele frequencies, thus differentiating the individuals of the new population genetically from the population of their ancestors. Salmonid populations in general are considered to be stable over long time (Ryman 1983; Nielsen et al. 1997; Laikre 1999). The observed allele frequency changes in the two tributaries are therefore likely to be a result of strong genetic drift created by a combination of fragmentation and, possibly, founder events following local extinctions.

There was a tendency for the incorrectly classified individuals of the contemporary populations to be assigned to the neighbouring population. In the historic populations there was a similar pattern as the number of individuals from the HOL40 sample assigned to the main river population SKJ40 were greater than the number assigned to VOR40 and vice versa. Thus, the assignment tests seemed to support that there is an effect of distance. The fact that we did not find any grayling upstream the Harrild water mill (Figure 1), as opposed to the findings of Ernst and Nielsen (1981) approximately 20 years earlier, does indeed indicate that even small weirs can have fatal effects on local populations by prohibiting recolonisa-

Table 5. Summary statistics for grayling data collected in 1940 and 1997/2000 based on the MSVAR program from Beaumont (1999). Estimates and standard deviations from 5 independent Markov Chains with different starting values analysed using the linear model. The parameter values (lower and upper bounds respectively) used in the simulations are shown in the first column. The parameters are scaled in terms of the current population size N_0: $r = N_0/N_1$, $t_f = t_a/N_0$ and $\theta = 2N_0\mu$. N_0 is the number of chromosomes present in the present population, N_1 is the number of chromosomes in the past population living some t_a generations ago before it began to decline, and μ is the microsatellite mutation rate (assumed to be fixed in our estimations)

			Contemporary populations		Historic populations	
Population	Parameter	Value	Estimate	Std. dev.	Estimate	Std. dev.
HOL	r	(−3.2)	0.00262	0.00009	0.00244	0.00043
	t_f	(−3.0)	32.50574	1.63123	31.94185	5.47818
	θ	(−2.1)	0.15066	0.01581	0.10282	0.02787
VOR	r	(−3.2)	0.00312	0.00045	0.00448	0.00021
	t_f	(−3.0)	25.22087	3.05575	19.94390	0.88753
	θ	(−2.1)	0.15241	0.03761	0.35618	0.05050
SKJ	r	(−3.2)	0.00327	0.00021	0.00351	0.00017
	t_f	(−3.0)	23.04517	1.89665	21.94219	0.64217
	θ	(−2.1)	0.42985	0.05237	0.17237	0.02286
FJE	r	(−3.2)	0.00254	0.00009		
	t_f	(−3.0)	32.56267	0.97264		
	θ	(−2.1)	0.12279	0.00987		

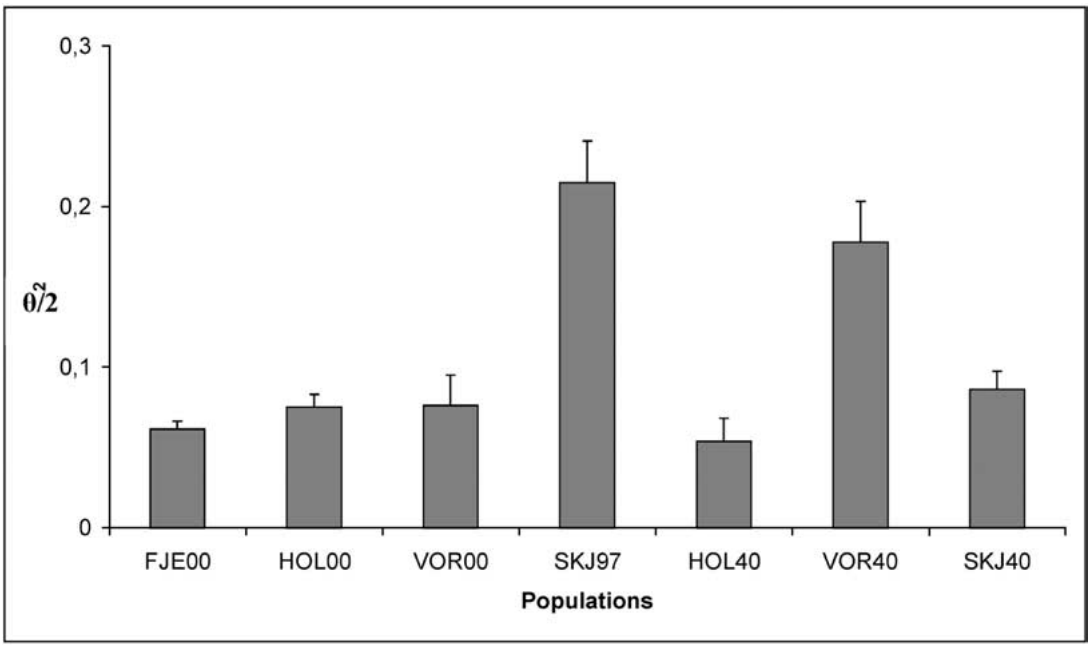

Figure 3. Relative population sizes (theta/2) with standard deviations based on five simulations with different starting points as estimated by MSVAR (Beaumont 1999).

tion. However, it is not clear what has caused the extinction of this population, as no polluting events are known to have happened in this time period.

The comparisons of relative population size estimates over time (Figure 3) indicated that the SKJ population had doubled since the forties. However, the lack of information on the exact location of the old sample sites could have biased the results of simulations, especially considering that samples from the SKJ40 population in theory could have been separated from each other by more than 80 kms of river distance. This might also have had an effect on the two other populations from the forties, but as these tributaries are much smaller, we expect the biases to be of lesser importance. The data suggest that the VOR population has undergone a reduction in size that is in agreement with our knowledge of the river's history of intensive pollution. To our knowledge, the Holtum River has not experienced pollution to the same extent as the Vorgod River, which is supported by the fact that there was no significant difference in N_0 estimates between HOL00 and HOL40.

The relative population size estimates from the present day populations fit our expectations as the main river estimate was three times larger than the ones from the tributaries. The fact that the FJE00 population estimate was significantly smaller than HOL00 and smaller than VOR00 is also in agreement with our preliminary knowledge of the populations, as a population decline was reported by local anglers. Furthermore, while sampling, we noticed long stretches of river with practically no fish, and overall we considered the FJE00 population to be the smallest of the four. Hence, we conclude that this approach is useful as a relative measure of differences in population size between populations. However, as pointed out by Beaumont (1999), results obtained by his method should be interpreted with some caution as the analysis assumes a strict SMM, which is probably invalid for microsatellite loci.

In order to look further into the effects of weirs on fish populations, it is necessary to keep the life history of the species in mind. Grayling differ from trout in their choice of habitat as they require a certain minimum size of river. This means that they enter smaller tributaries to a lesser extent than trout (Northcote 1995; Parkinson et al. 1999). Furthermore, grayling fry migrate or drift downstream within $1–1^1/_2$ months after hatching (Northcote 1995) as opposed to trout which normally spend at least one winter in their natal tributary (Elliott 1994). This makes grayling more exposed to pollutions since it has to re-colonise the polluted part of the river from a smaller number of upstream retreats than trout that can re-establish a population without any severe decline in genetic variation. If we consider two populations of grayling separated by an impassable weir, a fraction of the drifting fry will follow the water through the weir, thereby creating gene-flow from the upstream to the downstream population but not vice versa. Once downstream they cannot return to the upstream population, which leads to long-term flushing or unidirectional transport of individuals and alleles from the upstream population. Consequently, the effective size of the upstream population size is likely to decrease and genetic drift will increase leading to population differentiation. Under natural conditions, the early downstream drift of fry would be counteracted by upstream migration at later life stages.

The assignment tests showed a higher proportion of correctly assigned individuals in the tributaries than in the main river, implying larger distinctness of populations in the tributaries. This, along with the life history of grayling, suggests that the population structure of grayling consists of a main stem population, which continuously receives individuals and alleles from populations in the upstream situated tributaries and hence can be seen as a mixture of different populations. This view is supported by Bouvet et al. (1990), who found a similar pattern in grayling in the Rhone River system in France, where genetic variability was lower in the upper parts of the river system. The authors explained this observation solely as a result of higher selection pressure as the populations are marginally situated in the grayling zone of the river. This effect might be accompanied by a fragmentation effect as at least one of the tributaries was cut off from the main river by a dam.

Parkinson et al. (1999) found that spawning migrations of grayling in a Belgian river of a similar size and properties as the Skjern River tributaries, did not exceed five kms as opposed to resident trout in the same river (<25 kms). The authors concluded that as a consequence of these shorter spawning migration distances, weirs would have a spatially limited and lesser impact on resident grayling compared to brown trout. This might be true in large river systems or centrally situated grayling populations, but is probably not true in marginally upstream situated populations, where weirs act as one-way barriers allowing individuals to drift downstream but not to migrate upstream.

The assignment tests also revealed that the majority of the present Skjern River population were assigned to the historic Skjern River population highlighting the small genetic changes in this population. Similarly a relatively high proportion of the present Holtum River population were assigned to the historic Holtum River population. This indicates that even if the genetic composition has been significantly changed, the present grayling population in Holtum River are most likely descendants of the historic population. For the Vorgod River, however, few fish from the present population are assigned (and many rejected) from the historic population while many are assigned to the historic Skjern River population. This could have several causes. First of all the historic Vorgod sample is quite small (n = 13), which makes estimation of allele frequencies somewhat uncertain and could bias the results of the assignment tests. Also, this could be due to the uncertainty of the exact location of sampling sites in the 1940 populations as the sampling site of historic Skjern River individuals could, supposedly, have been close to mouth of the Vorgod River, which lies approximately 20 kilometres from the sampling site of the contemporary sample. Finally, the Vorgod River experienced a severe pollution event at some point between 1940 and 2000 that wiped out the majority of the population, followed by a recolonisation from the Skjern River. Considering the river's known history of severe pollution this explanation seems highly likely.

The drastic long-term population decline suggested by the results applying Beaumont's (1999) method is likely to be a result of postglacial events. The Skjern River system was formed 10,000–13,000 years ago. At that time the water level of the North Sea was low and the Skjern River was a tributary to the Elbe River system, which ran in a North-Westerly direction (Nielsen 1975). As described by Hansen et al. (1999) freshwater fish species like grayling and dace (*Leusiscus leusiscus*) entered the Skjern River system from the southern parts of the Elbe River. The water level of the North Sea gradually increased and from approximately 7500 years ago the rivers of western Jutland became disconnected from the Elbe River, thereby abstricting the populations of the Skjern River system from the Elbe populations and other of the former tributaries. As grayling is a saltwater intolerant species, the abstriction combined with founder events might have led to the decrease in population size proposed by Beaumont's (1999) approach. It is, however, unfortunate that the detection of recent bottlenecks is swamped by this massive population decline.

In summary, our study supports the hypothesis that weirs can have a fragmentation effect on the genetic structure of grayling. There were signs supporting the hypothesis that impassable weirs speed up genetic drift, which increases the probability of losing rare alleles, and thereby lowering genetic variability. Haugen and Vøllestad (2000) showed that grayling populations, which shared common ancestors 80–90 years ago (13–18 generations), were able to adapt to different temperature regimes within this, in an evolutionary context, relatively short time period. Likewise, Koskinen et al. (2002) demonstrated life-history evolution in small populations of grayling that originated from a common ancestor 80-120 years ago. To facilitate similar adaptations in the future, it is of great importance to maintain the genetic variability of the species. However, in our study, there were no signs of an overall loss of genetic variability, supporting that the population structure of grayling makes the species resilient to local population declines or even local extinctions as long as these extinctions do not take place in fragmented upper parts of tributaries.

Conservation and management implications

Our study provides useful information on how to manage threatened populations of grayling. First of all our data underlines the importance of providing efficient bypasses designed to enable upstream migration of grayling. Otherwise this can lead to disruption of the natural genetic population structure of the species. Secondly, in situations where local extinctions have taken place and stocking is considered, it is important to stock with fish from neighbouring populations, since marked genetic differentiation among populations within a river system can be found. If one has to choose between stocking with fish from the main stem and fish from another tributary, we support stocking with fish from the main stem, due to the supposed higher genetic variability of main stem populations and the ensuing higher probability that they will successfully adapt to the environment.

Hence, conservation efforts on grayling should resemble those applied to other salmonid species such as trout, i.e. they should focus on improving environmental conditions, possibly supplemented with supportive breeding of local populations (Hansen et al. 2001a and references therein). Although not as intensively as with e.g. trout, stocking with domesti-

cated grayling is currently performed in both North America and Europe (Northcote 1995). Several studies have documented that stocking with domesticated trout should be avoided from a conservation point of view as hatchery individuals seem to have lower long-term fitness in the wild (Hansen et al. 2000; Hansen et al. 2001a; Hansen et al. 2001b; Poteaux et al. 1998; Ruzzante et al. 2001). If there is an effect at all from stocking, it is likely to be negative, causing introgression and possibly outbreeding depression (Hindar et al. 1991b). It is also important to bear this in mind for the conservation of grayling by avoiding stocking with exogenous fish from domesticated and foreign strains. Instead local broodstock should be used.

Acknowledgements

We thank the Danish Institute For Fisheries Research (DIFRES) and the Danish Natural Science Research Council for financial support, Michael Møller Hansen for many useful suggestions, Karen-Lise Mensberg and Dorte Meldrup for technical assistance, Thorbjørn Søndergaard, Søren Widt and Anders Bertelsen for assistance with electrofishing and Cino Pertoldi for helpful advice on the use of the Beaumont program. VL also wants to thank the Institute for Advanced Study at La Trobe University for their hospitality, where he was fellow when the final version of the ms was written and the Center for Environmental Stress and Adaptation Research (CESAR).

References

Beaumont MA (1999) Detecting population expansion and decline using microsatellites. *Genetics*, **153**, 2013–2029.

Bouvet Y, Soewardi K, Pattee E (1990) Genetic divergence within natural populations of grayling (*Thymallus thymallus*) from two French river systems. *Arch. Hydrobiol*., **119**, 89–101.

Carlson J, Olsén KH, Nilsson J, Øverli Ø, Stabell OB (1999) Microsatellites reveal fine-scale genetic structure in stream-living brown trout. *Journal of Fish Biology*, **55**, 1290–1303.

Carlsson J, Nilsson J (2001) Effects of geomorphological structures on genetic differentiation among brown trout populations in a northern boreal river drainage. *Transactions of the American Fisheries Society*, **130**, 36–45.

Carvalho GR, Hauser L (1998) Advances in the molecular analysis of fish population structure. *Ital. J. Zool*., **65** (Suppl.), 21–33.

Cornuet JM, Piry S, Luikart G, Estoup A, Solignac M (1999) Comparison of methods employing multilocus genotypes to select or exclude populations as origins of individuals. *Genetics*, **153**, 1989–2000.

Elliott JM (1994) *Quantitative Ecology and the Brown Trout*. Oxford: Oxford University Press.

Ernst, ME, Nielsen J (1981) *Populationsdynamiske undersøgelser over* Stalling *(*Thymallus thymallus *(L.) i øvre Gudenå*. MS thesis, University of Aarhus, Denmark (In Danish).

Estoup A, Largiadèr CR, Perrot E, Chourrot D (1996) Rapid one-tube DNA extraction for reliable PCR detection of fish polymorphic markers and transgenes. *Molecular Marine Biology and Biotechnology*.

Estoup A, Rousset F, Michalakis Y, Cornuet J-M, Adriamanga M, Guyomard R (1998) Comparative analysis of microsatellite and allozyme markers: A case study investigating microgeographic differentiation in brown trout (*Salmo trutta*). *Molecular Ecology*, **7**, 339–353.

Goudet J (2000) FSTAT, a program to estimate and test gene diversities and fixation indices (version2.9.1). Available from http://www.unil.ch/izea/softwares/fstat.html.

Guo SW, Thompson EA (1992) Performing the exact test for Hardy-Weinberg proportions for multiple alleles. *Biometrics*, **48**, 361–372.

Hansen MM, Loeschcke V (1996) Temporal variation in mitochondrial DNA haplotype frequencies in a brown trout (*Salmo trutta* L.) population that shows stability in nuclear allele frequencies. *Evolution*, **50**, 454–457.

Hansen MM, Mensberg K-L, Berg S (1999) Postglacial recolonisation patterns and genetic relationships among whitefish (*Coregonus sp*.) populations in Denmark, inferred from mitochondrial DNA and microsatellite markers. *Molecular Ecology*, **8**, 239–252.

Hansen MM, Ruzzante DE, Nielsen EE, Mensberg, K-L (2000) Microsatellite and mitochondrial DNA polymorphism reveals life-history dependent interbreeding between hatchery and wild brown trout (*Salmo trutta L.*). *Molecular Ecology*, **9**, 583–594.

Hansen MM, Nielsen EE, Bekkevold D, Mensberg K-L (2001a) Application of assignment methods for estimating the genetic impact of stocked domesticated brown trout (*Salmo trutta*) on wild trout populations (in press).

Hansen MM, Ruzzante DE, Nielsen EE, K-L Mensberg (2001b) Brown trout (*Salmo trutta*) stocking impact assessment using microsatellite DNA markers. *Ecol. Appl*., **11**, 148–160.

Haugen TO, Vøllestad LA (2000) Population differences in early life-history traits in grayling. *J. Evol. Biology*., **13**, 897–905.

Hedrick PW, Miller PS (1992) Conservation genetics: Techniques and fundamentals. *Ecological Applications*, **2**, 30–46.

Hindar K, Jonsson B, Ryman N, Ståhl G (1991a) Genetic relationships among landlocked, resident, and anadromous brown trout, *Salmo trutta L. Heredity*, **66**, 83–91.

Hindar K, Ryman N, Utter FM (1991b) Genetic effects of cultured fish on natural fish populations. *Can. J. Aquat. Sci*., **48**, 945–957.

Jungwirth M 1996. Bypass channels at weirs as appropriate aids for fish migration in Rhithral rivers. *Regulated Rivers Research and Management*, **12**, 483–492.

Koskinen MT, Primmer CR (1999) Cross-species amplification of salmonid microsatellites which reveal polymorphism in European and Arctic grayling, *Salmonidae: Thymallus spp*. *Hereditas*, **131**, 171–176.

Koskinen MT, Ranta E, Piironen J, Veselov A, Titov S, Haugen TO, Nilsson J, Carlstein M, Primmer CR (2000) Genetic lineages and postglacial colonization of grayling (*Thymallus thymallus, Salmonidae*) in Europe, as revealed by mitochondrial DNA analyses. *Molecular Ecology*, **9**, 1609–1624.

Koskinen MT, Haugen TO, Primmer CR (2002) Contemporary fisherian life-history evolution in small salmonid populations. *Nature*, **419**, 826–830.

Laikre L (ed.) (1999) Conservation genetic management of brown trout (*Salmo trutta*) in Europe. Report by the concerted action on

identification, management and exploitation of genetic resources in the brown trout ("Troutconcert"; EU fair CT97-3882).

Mantel N (1967) The detection of disease clustering and a generalised regression approach. *Cancer Research*, **27**, 209–220.

Miller LM, Kapuscinski AR (1997) Historical analysis of genetic variation reveals low effective population size in a northern pike (*Esox lucius*) population. *Genetics*, **147**, 1249–1258.

Nielsen AV (1975) Landskabets tilblivelse. In: *Danmarks Natur*, Landskabernes opståen (eds. Nørrevang A, Meyer TJ), pp. 251–344. Politikens Forlag, Copenhagen.

Nielsen EE, Hansen MM, Loeschcke V (1997) Analysis of microsatellite DNA from old scale samples of Atlantic salmon: A comparison of genetic composition over sixty years. *Molecular Ecology*, **6**, 487–492.

Nielsen EE, Hansen MM, Loeschcke V (1999a) Genetic variation in time and space: Microsatellite analysis of extinct and extant populations of Atlantic salmon. *Evolution*, **53**, 261–268.

Nielsen EE, Hansen MM, Loeschcke V (1999b) Analysis of DNA from old scale samples: Technical aspects, applications and perspectives for conservation. *Hereditas*, **130**, 265–276.

Northcote TG (1995) Comparative biology and management of Arctic and European grayling (*Salmonidae, Thymallus*) *Reviews in Fish Biology and Fisheries*, **5**, 141–194.

Olsen JB, Bentzen P, Seeb JE 1998. Characterization of seven microsatellite loci derived from pink salmon. *Molecular Ecology*, **7**, 1083–1090.

Paetkau D, Calvert W, Sterling I, Strobeck C (1995) Microsatellite analysis of population structure in Canadian polar bears. *Molecular Ecology*, **4**, 347–354.

Parkinson D, Philippart J-C, Baras B (1999) A preliminary investigation of grayling in a small stream as determined by radiotracking. *Journal of Fish Biology*, **55**, 155–182.

Piry S, Bouget C (1999) POP100GENE (version 1.1.02): http://www.ensam.inra.fr/URLB/pop100gene/pop100gene.html

Poteaux C, Beaudou D, Berrebi P (1998) Temporal variations of genetic introgression in stocked brown trout populations. *Journal of Fish Biology*, **53**, 701–713.

Purcell M, Kornfield I, Fogarty M, Parker A (1996) Interdecadal heterogeneity in mitochondrial DNA of Atlantic haddock (*Melanogrammus aeglefinus*) from Georges Bank. *Molecular Marine Biology and Biotechnology*, **5**, 185–192.

Rannala B, Mountain JL (1997) Detecting immigration by using multilocus genotypes. *Proceedings of the National Academy of Sciences USA*, **94**, 9197–9201.

Raymond M, Rousset F (1995a) GENEPOP: Population genetics software for exact tests and ecumenism. Vers. 1.2. *J. Hered.*, **86**, 248–249.

Raymond M, Rousset F (1995b) An exact test for population differentiation. *Evolution*, **43**, 223–225.

Redenbach Z, Taylor EB (1999) Zoogeografical implications of variation in mitochondrial DNA of Arctic grayling (*Thymallus Arcticus*). *Molecular Ecology*, **8**, 23–35.

Rice WR (1989) Analysing tables of statistical tests. *Evolution*, **43**, 223–225.

Rousset F (1997) Genetic differentiation and estimation of gene flow from *F*-statistics under isolation by distance. *Genetics*, **145**, 1219–1228.

Ruzzante DE (1998) A comparison of several measures of genetic distance and population structure with microsatellite data: Bias and sampling variance. *Can. J. Fish Aquat. Sci.*, **55**, 1–14.

Ruzzante DE, Hansen MM, Meldrup D (2001) Distribution of individual inbreeding coefficients, relatedness and influence of stocking on native anadromous brown trout (*Salmo trutta*) population structure. *Molecular Ecology*, **10**, 2107–2128.

Ryman N (1983) Patterns of distribution of biochemical genetic variation in salmonids: Differences between species. *Aquaculture*, **33**, 1–21.

Sakamoto T, Okamoto N, Ikeda U, Nakamura Y, Sato T (1994) Dinucleotide-repeat polymorphism in DNA of rainbow trout and its application in fisheries science. *Journal of Fish Biology*, **44**, 1093–1096.

Scribner KT, Gust JR, Fields RL (1996) Isolation and characterization of novel salmon microsatellite loci: Cross-species amplification and population genetic applications. *Can. J. Fish Aquat. Sci.*, **53**, 833–841.

Skaala Ø, Nævdal G (1989) Genetic differentiation between freshwater resident and anadromous brown trout, *Salmo trutta*, within watercourses. *Journal of Fish Biology*, **34**, 597–605.

Slatkin M (1995) A measure of population subdivision based on microsatellite allele frequencies. *Genetics*, **139**, 457–462.

Sušnik S, Snoj A, Dovc P (1999a) Microsatellites in grayling (*Thymallus thymallus*): Comparison of two geographically remote populations from the Danubian and Adriatic river basin in Slovenia. *Molecular Ecology*, **8**, 1756–1758.

Sušnik S, Snoj A, Dovc P (1999b) A new set of microsatellite markers for grayling: BFRO014, BFRO015, BFRO016, BFRO017 and BFRO018. 1999. *Animal Genetics*, **30**, 462–478.

Sušnik S, Snoj A, Jesenšek D, Dovc P (2000) Microsatellite DNA Markers (BFRO010 and BFRO011) for grayling. *J. Anim. Sci.*, **78**, 488–489.

Taggart JB, Hynes RA, Prodöhl PA, Ferguson A (1992) A simplified protocol for routine total DNA isolation from salmonid fishes. *Journal of Fish Biology*, **40**, 963–965.

Tessier N, Bernatchez L (1999) Stability of population structure and genetic diversity across generations assessed by microsatellites among sympatric populations of landlocked Atlantic salmon (*Salmo salar* L.). *Molecular Ecology*, **8**, 169–179.

Vriejenhoek RC (1994) Genetic diversity and fitness in small populations. In: *Conservation Genetics* (eds. Loeschcke V, Tomiuk J, Jain SK), pp. 37–53. Birkhäuser, Basel.

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