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RESEARCH ARTICLE

Historical and recent genetic bottlenecks in European grayling, *Thymallus thymallus*

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Abstract Sharp declines in population size, known as genetic bottlenecks, increase the level of inbreeding and reduce genetic diversity threatening population sustainability in both short- and long-term. We evaluated the presence, severity and approximate time of bottlenecks in 34 European grayling (*Thymallus thymallus*) populations covering the majority of the species distribution using microsatellite markers. We identified footprints of population decline in all grayling populations using the *M* ratio test. In contrast to earlier simulation studies assuming isolated populations, forward simulations allowing low levels of migration demonstrated that bottleneck footprints measured using the *M* ratio can persist within small populations much longer (up to thousands of generations) than previously anticipated. Using a coalescence approach, the beginning of population reduction was dated back to 1,000–10,000 years ago which suggests that the extremely low *M* ratio in European grayling is most likely caused by the last glaciation and subsequent post-glacial recolonization processes. In contrast to the *M* ratio, two alternative methods for bottleneck detection identified more recent bottlenecks in six populations and thus, from a conservation perspective, these populations warrant future monitoring. Based on a single time-point analysis using approximate Bayesian computation methodology, all grayling populations exhibited very small effective population sizes with the majority of N_e estimates below 50. Taken together, our results demonstrate the predominate role of genetic drift in European grayling populations in the short term but also emphasize the importance of gene flow counteracting the effects of genetic drift and loss of variation over longer evolutionary timescales.

Keywords *Thymallus thymallus* · Genetic bottleneck · *M* ratio · Heterozygosity excess test · Mode-shift test · Microsatellite

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Introduction

Reductions in population size by several orders of magnitude often occur in nature. Such population size declines can result in the loss of genetic diversity (Frankham 1996; Houlden et al. 1996) and fixation of alleles (Lande 1994) that can lower the likelihood of short-term population existence, as descendants of small populations are more likely to suffer from inbreeding depression (Frankham 1995; Saccheri et al. 1998) and are more susceptible to various diseases and parasites (Hale and Briskie 2007). The ability of a population to respond to environmental changes is also decreased in the long-term (Frankham 1995). As a result, identification of drastic population declines remains one of the important issues in conservation biology.

Currently, molecular genetic methods enable the detection of bottleneck footprints (Rand 1996; Luikart et al. 1998b) over different time periods ranging from tens to hundreds of generations (Cornuet and Luikart 1996; Luikart et al. 1998a; Beaumont 1999; Garza and Williamson 2001). An increasing number of studies have detected such bottleneck footprints in various species groups, including insects (Dhuyvetter et al. 2005; Chen et al. 2006), mollusks (Bouza et al. 2007), amphibians (Beebee and Rowe 2001),

reptiles (Russello et al. 2007), birds (Lambert et al. 2005), fish (Vasemägi et al. 2005; Vähä et al. 2007) and mammals (Lucchini et al. 2004; Fernandez-Stolz et al. 2007). However, it has been shown that bottlenecks generate detectable genetic footprints only in extreme cases when effective population sizes decreases rapidly to tens rather than to hundreds of individuals (Luikart and Cornuet 1998). As a result, many studies have failed to detect bottleneck footprints from genetic data even when demographic data indicate that the population has gone through size collapse (Le Page et al. 2000; Queney et al. 2000; Busch et al. 2007; Steinfartz et al. 2007; Mardulyn et al. 2008). On the other hand, populations with rather high census sizes can exhibit footprints of 'cryptic' genetic bottlenecks since rapid reduction in effective population size does not necessarily co-occur with drastic reductions in census size (Briscoe et al. 1992; Luikart et al. 1998a). Moreover, the strength of the bottleneck footprint depends on multiple factors including duration (Nei et al. 1975) and the time elapsed since the population reduction (Maruyama and Fuerst 1985), which relate to the generation time of the studied species (Dinerstein and McCracken 1990). As a result, it is not surprising that only a relatively small number of studies to date have found strong footprints of genetic bottlenecks in natural populations (Abdelkrim et al. 2005; Vasemägi et al. 2005; Wang et al. 2005; Spear et al. 2006).

Genetic bottlenecks are fundamentally linked to effective population size (N_e), a central population genetic parameter relevant for conservation. The effective population size can be estimated using genetic approaches based on estimation of linkage disequilibrium (Hill 1981), heterozygote excess (Pudovkin et al. 1996; Luikart and Cornuet 1999), temporal changes in allele frequency (Waples 1989) or coalescence methods (Beerli and Felsenstein 2001). These approaches are frequently used for conservation and monitoring of both wild and captive populations (Schwartz et al. 1998; Fraser et al. 2007). Still, it has been suggested that N_e estimates derived from a single time-point should be regarded with caution, especially when sample sizes are small (England et al. 2006; Waples 2006). However, a new approach has been recently developed that combines multiple genetic parameters, such as, linkage disequilibrium, expected heterozygosity, number of alleles per locus and mean and variance of multilocus homozygosity, for estimation of N_e using an approximate Bayesian computation framework (Tallmon et al. 2004). This should lead to increase both accuracy and precision of the N_e estimates, but to date, this approach has been applied in only a few natural populations (Witzenberger and Hochkirch 2008; Aspi et al. 2009; Johnson et al. 2009).

European grayling, *Thymallus thymallus*, a salmonid fish native to Europe, is a popular target for recreational anglers. The majority of populations inhabit freshwater while some populations occupy brackish water in the northern Baltic Sea. Many grayling populations currently suffer from habitat fragmentation and environmental degradation caused by various anthropogenic activities. As a consequence, many grayling populations are threatened (Uiblein et al. 2001; Duftner et al. 2005) and are listed as a protected species in appendix III of the Bern Convention. Earlier phylogeographic studies on grayling have demonstrated that postglacial colonization of northern Europe was from central and eastern European refugia (Koskinen et al. 2000). Grayling populations exhibit high levels of genetic divergence also at smaller geographical scales. For example, differentiation among populations from two adjacent drainages, the Rhine/Main and the Danube, was very high as revealed by microsatellite markers ($F_{ST} = 0.369$; Gum et al. 2003). High differentiation ($F_{ST} = 0.261$) has also been found among populations residing in the same lake, indicating very low levels of gene flow across short waterway distances (Koskinen et al. 2001). Grayling exhibit very low overall genetic diversity (Koskinen et al. 2002c; Susnik et al. 2004; Gum et al. 2006) compared to other salmonids (e.g. Vasemägi et al. 2005; Gross et al. 2007; Fave and Turgeon 2008; Lehtonen et al. 2009). The population structure of grayling has been recently characterized throughout Finland and three previously unanticipated population groups have been described, corresponding to the northern, Baltic and southeastern geographic regions (Swatdipong et al. 2009). However, despite the fact that the population structure of grayling has been extensively studied (Gross et al. 2001; Susnik et al. 2001; Koskinen et al. 2002a, d), only a single genetic footprint of recent population decline has been reported in a European grayling population occupying the Rhine basin, Germany (Gum et al. 2003).

The major aim of this study was to systematically screen for genetic bottlenecks in European grayling using microsatellite data from 34 populations collected from northern Europe (Denmark, Norway, Sweden, Finland), Russia, and central Europe (Germany and Slovenia) using four published datasets (Koskinen et al. 2002c; Gum et al. 2003; Korkea-Aho 2003; Swatdipong et al. 2009). We also performed a series of population genetic simulations to evaluate how long the footprints of bottlenecks, measured using the *M* ratio (Garza and Williamson 2001), persist within a population in the face of gene flow. In addition, we estimated the time of the bottlenecks using a coalescent approach and the N_e in an approximate Bayesian computation framework.

Materials and methods

Combination of independent microsatellite datasets

The genotype datasets used in the present study were derived from three previous studies (Koskinen et al. 2002c;

Korkea-Aho 2003; Swatdipong et al. 2009) consisting a total of 30 grayling populations. Allele frequencies and allele sizes of microsatellite loci genotyped in four wild grayling populations from Germany were additionally obtained from Gum et al. (2003). The majority of the samples (19 populations) were from Finland, three each from Russia and Sweden, two from Germany and one population from Norway, Denmark and Slovenia (Fig. 1). All samples represent indigenous populations except for three cases: Isojoki and Rauanjoki populations are of mixed origin according to Finnish fishery records (Kaukoranta et al. 2000) and Lesjaskogsvatn from Norway

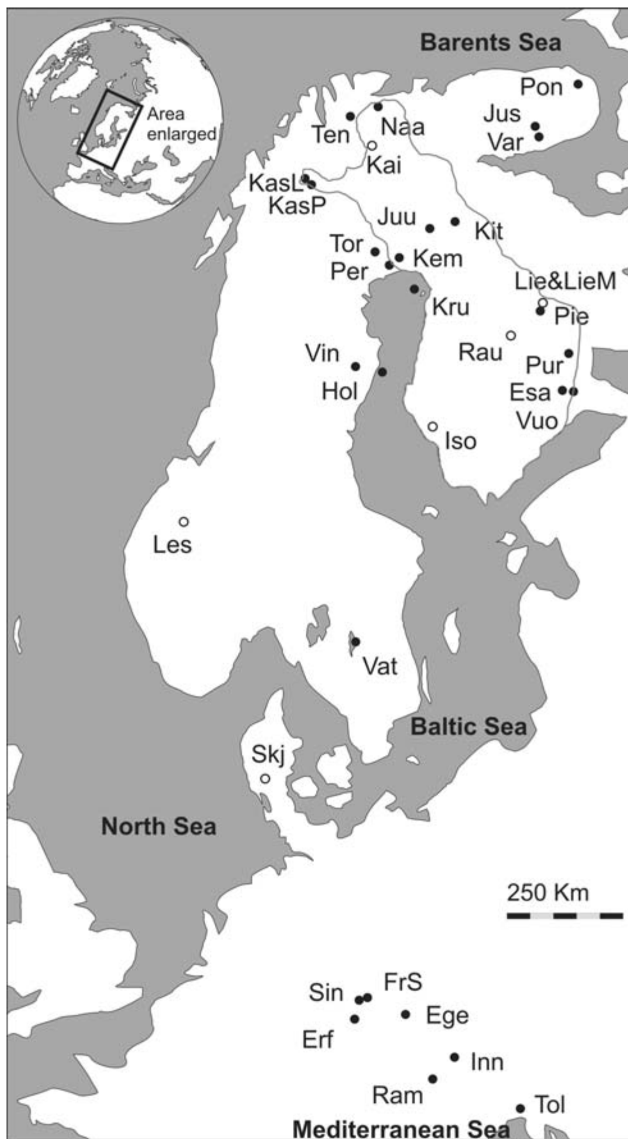

Fig. 1 Locations of the studied European grayling populations ranging over 2800 km from the Mediterranean to the Barents Sea basin. Open dots indicate six populations exhibiting the footprints of recent population decline. Populations are coded as in Table 1

represents an introduced population, as documented by Koskinen et al. (2002a). Population information and abbreviations are in Table 1.

Twenty-five populations were previously genotyped using a set of 13 microsatellite loci (Swatdipong et al. 2009). Five wild populations; Pon (Korkea-Aho 2003), Ram, Skj, Tol and Var (Koskinen et al. 2002c); were included after calibrating allele sizes with the first 25 populations. As in the first dataset, populations from the two latter datasets were screened for identification of migrants based on their multilocus genotype using STRUCTURE 2.2 (Pritchard 2000; see detail in Swatdipong et al. 2009), but none were identified. The combined genotype dataset comprised 1,172 individuals from 30 populations. Ten microsatellites were genotyped in all populations (BFRO5, BFRO10, BFRO11, BFRO12, BFRO15, BFRO17, BFRO18, Ogo2, Str73INRA and Str85INRA), while five additional loci (BFRO7, BFRO9, BFRO13, BFRO16 and Cocl23) were genotyped only in a subset of populations. Details regarding allele size calibration by re-genotyping of known individuals are available in Swatdipong et al. (2009). Data for four wild grayling populations in Germany (Inn from Danube; Erf, FrS and Sin from Rhine) were accessed through the supplementary appendix of Gum et al. (2003). We excluded the loci BFRO4 and One2 of their dataset from further analysis as both loci exhibited null alleles in several Finnish grayling populations (heterozygote deficiency test; $P < 0.05$ in six populations for BFRO4; while $P = 0.02$ in KasL and $P < 0.0001$ in three populations for One2; Swatdipong et al. 2009). BFRO5 was also excluded due to inconsistency in microsatellite repeat units. All microsatellite loci analyzed in the present study consisted of dinucleotide repeats. The genotype data from the first 30 populations were used in all analyses while allele frequency and allele data from four additional German populations were used only for calculation of the M ratio (Garza and Williamson 2001) and estimating the approximate time of the bottleneck (see below).

Statistical analyses

Microsatellite diversity, Hardy–Weinberg and linkage equilibrium

Allelic richness was measured at the 10 common microsatellite loci in 30 populations (rarefaction = 16 diploid individuals) using FSTAT 2.9.3.2 (Goudet 1995). Deviations from Hardy–Weinberg and linkage equilibria were tested using Genepop 3.4 (Raymond and Rousset 1995), based on 12–14 genotyped loci per population. Sequential Bonferroni correction (Holm 1979) was employed to account for multiple testing.

Table 1 Sample information, microsatellite diversity estimates and bottleneck test statistics in European grayling

Water system	Code	Location	Sample size	Microsatellite diversity		Bottleneck test statistics		
				Polymorphic loci (loci genotyped)	A_r	Wilcoxon's test^{SMM}	Wilcoxon's test^{TPM}	Mode-shift test
Tenojoki	Ten[a]	Finland, North	42	11 (13)	2.86	0.966^{NS}	0.949^{NS}	L-shape
Näätämöjoki	Naa[a]	Finland, North	35	11 (13)	2.82	0.768^{NS}	0.650^{NS}	L-shape
Kaitamo (Inari)	Kai[a]	Finland, North	34	6 (13)	1.73	0.055^{NS}	0.039*	Shifted
Kässivarsi, Poroeno	KasP[a]	Finland, North	20	11 (13)	2.97	0.990^{NS}	0.990^{NS}	L-shape
Kässivarsi, Lätäseno	KasL[a]	Finland, North	29	12 (13)	3.36	0.995^{NS}	0.993^{NS}	L-shape
Tornionjoki	Tor[a]	Finland, North	63	13 (13)	3.75	1.000^{NS}	0.999^{NS}	L-shape
Juujarvi	Juu[a]	Finland, North	35	12 (13)	2.53	0.993^{NS}	0.993^{NS}	L-shape
Kemijoki	Kem[a]	Finland, North	35	13 (13)	3.59	0.892^{NS}	0.863^{NS}	L-shape
Kitkajärvi	Kit[a]	Finland, North	67	13 (13)	3.13	0.294^{NS}	0.170^{NS}	L-shape
Perämeri	Per[a]	Finland, North	17	11 (13)	3.28	0.449^{NS}	0.382^{NS}	L-shape
Ulkokrunnit	Kru[a]	Finland, Bothnian bay	40	13 (13)	3.47	0.878^{NS}	0.812^{NS}	L-shape
Isojoki	Iso[a]	Finland, South-West	36	11 (13)	2.63	0.074^{NS}	0.034*	L-shape
Lieksanjoki	Lie[a]	Finland, South-East	36	12 (13)	2.20	0.311^{NS}	0.259^{NS}	Shifted
Lieksanjoki	LieM[a]	Finland, South-East	48	12 (13)	2.45	0.883^{NS}	0.849^{NS}	L-shape
Pielinen	Pie[a]	Finland, South-East	42	12 (13)	2.81	0.339^{NS}	0.285^{NS}	L-shape
Rauanjoki	Rau[a]	Finland, South-East	35	12 (13)	3.19	0.039*	0.026*	Shifted
Puruvesi	Pur[a]	Finland, South-East	36	13 (13)	3.41	0.554^{NS}	0.500^{NS}	L-shape
Etelä-Saimaa	Esa[a]	Finland, South-East	48	12 (13)	2.77	0.425^{NS}	0.311^{NS}	L-shape
Vuoksi	Vuo[a]	Finland, South-East	22	11 (13)	2.58	0.681^{NS}	0.650^{NS}	L-shape
Juzija river	Jus[a]	Russia, Kola peninsula	32	12 (13)	3.19	0.849^{NS}	0.849^{NS}	L-shape
Varzugar	Var[b]	Russia, Kola peninsula	26	12 (14)	3.16	0.715^{NS}	0.689^{NS}	L-shape
Ponoi	Pon[c]	Russia, Kola peninsula	112	10 (12)	2.75	0.997^{NS}	0.995^{NS}	L-shape
Vindelälven	Vin[a]	Sweden, East	38	11 (13)	3.26	0.650^{NS}	0.483^{NS}	L-shape
Holmön	Hol[a]	Sweden, Bothnian bay	34	13 (13)	4.51	0.207^{NS}	0.108^{NS}	L-shape
Vättern	Vat[a]	Sweden, South	45	12 (13)	3.23	0.633^{NS}	0.545^{NS}	L-shape
Lesjaskogsvatn	Les[a]	Norway, South	30	7 (13)	1.70	0.055^{NS}	0.055^{NS}	Shifted
Skjern	Skj[b]	Denmark, West	33	13 (13)	3.99	0.073^{NS}	0.040*	L-shape
Tolminka	Tol[b]	Slovenia, West	28	13 (14)	4.07	0.108^{NS}	0.055^{NS}	L-shape
Eger, Elbe	Ege[a]	Germany, Central	37	11 (13)	3.13	0.961^{NS}	0.867^{NS}	L-shape
Ramsach, Danube	Ram[b]	Germany, South	37	13 (14)	4.77	0.987^{NS}	0.953^{NS}	L-shape
Erf, Rhine	Erf[d]	Germany, South	15	15	–	–	–	–
Fr. Saale, Rhine	FrS[d]	Germany, South	15	13	–	–	–	–
Sinn, Rhine	Sin[d]	Germany, South	39	14	–	–	–	–

Table 1 continued

Water system	Code	Location	Sample size	Microsatellite diversity		Bottleneck test statistics		
				Polymorphic loci (loci genotyped)	A_r	Wilcoxon's test[SMM]	Wilcoxon's test[TPM]	Mode-shift test
Inn, Danube	Inn[d]	Germany, South	11	15	–	–	–	–

Allelic richness (A_r) is averaged from 10 microsatellite loci genotyped across all populations

[NS] Non-significant ($P > 0.05$)

*$0.01 < P \leq 0.05$ (without sequential Bonferroni correction)

[a, b, c, d] Data originating from Swatdipong et al. (2009), Koskinen et al. (2002c), Korkea-Aho (2003) and Gum et al. (2003), respectively

M ratio test

To detect genetic bottlenecks that occurred over relatively long periods of time (>100 generations), the M ratio test (Garza and Williamson 2001) that utilizes the stepwise nature of microsatellite mutations for bottleneck detection was used. The M ratio for each locus was manually calculated based on the equation of Garza and Williamson (2001) using a slight modification according to Excoffier et al. (2005) as implemented in Arlequin 3.11 (Schneider et al. 2000). Specifically, all monomorphic loci within a particular population were excluded, since loci with fixed alleles can erroneously increase the average M ratio upwards. In this study, the M ratio of a population was thus calculated using only polymorphic loci. Ninety-five percent confidence intervals (CI) of the M ratio were obtained by re-sampling with replacement over loci for 10,000 replications using the Excel add-in program PopTools 2.7.5 (Hood 2006).

To minimize the likelihood of type I errors (i.e. erroneously claiming a bottleneck when there is none) the population specific parameter M_c (Garza and Williamson 2001) was calculated with the mean size of non-stepwise mutations = 3.5 and $\theta = 10$ as suggested by Garza and Williamson (2001). For the first setting, the proportion of stepwise mutations (stepwise mutation model, SMM) was set to 90% as recommended by Garza and Williamson (2001) while in the second setting the proportion of single-step mutations was 80%. For calculation of M_c, 10,000 simulation replicates were run using Critical M (Garza 2006).

Wilcoxon's test and mode-shift test

To detect more recent genetic bottlenecks, two tests were used: the Wilcoxon's sign rank test which is based on heterozygosity excess and the mode-shift test which evaluates the allele frequency distribution. These bottleneck footprints are detectable over a relatively short period, ca. $<4N_e$ generations for heterozygosity excess and only a few dozen generations for the mode-shift test (Cornuet and Luikart 1996).

Both bottleneck tests were performed using BOTTLENECK 1.2.02 (Piry et al. 1999) using both the stepwise mutation model (SMM) and the two-phase model (TPM) as recommended by Luikart and Cornuet (1998). It has been previously demonstrated that the mutation model has a strong effect on the estimation of heterozygosity excess, but not on the distortion of allele frequency distribution since rare alleles are expected to be abundant regardless of the mutation model (Nei et al. 1976). The TPM comprised 95% single step mutations and 5% multi-step mutations for which the variance for mutation size was set to 12 as suggested by Piry et al. (1999). Altogether 100,000 simulations were run. For the Wilcoxon's sign rank test, 95% SMM can be considered as a relatively conservative setting for bottleneck detection, providing a reasonable balance between type I and type II error (Williamson-Natesan 2005).

Dating past demography

A coalescent analysis approach was used to estimate the approximate time of the decrease in population size based on microsatellite allele counts and allele sizes (Beaumont 1999; Storz and Beaumont 2002). We utilized a Markov Chain Monte Carlo simulation approach based on a hierarchical Bayesian model for the coalescence analysis, implemented in MSVAR 1.3 assuming strict SMM (no need to provide prior mutation rate; Beaumont 2004). For the simulations, generation time of grayling was set to 5 years, as previously used by Koskinen et al. (2002b), falling within the range (4.01–7.45 years per generation) estimated by Haugen and Vøllestad (2001). Change in population size was evaluated using both linear and exponential models, for comparative purposes. The exponential model was initially suggested to accurately date the change of population size in a relatively short timescale while the linear model was better suited for a longer timescale (Beaumont 1999). However, the exponential

model in the current version of MSVAR is also suitable for estimation change in population size over relatively long periods of time (Storz and Beaumont 2002; Storz et al. 2002). Other parameters were set as recommended in the MSVAR manual. The simulations were run for 2×10^8 iterations (output number = 20,000 lines, iterations number between outputs = 10,000). The first 10% of the output (2,000 lines) was discarded to alleviate possible bias from the starting value. Hence, the only 90% of output was used for subsequent analysis.

Effect of migration on the M ratio

Based on population genetic simulations, it has been previously demonstrated that genetic bottleneck signatures expressed as low *M* ratio can persist for approximately 100–300 generations when the population remains isolated (Garza and Williamson 2001). However, the effect of migration on the *M* ratio has not been rigorously tested. Here, we used simple forward simulations to specifically evaluate how migration affects the *M* ratio after a reduction in effective population size from 10,000 to 50 (permanent bottleneck scenario) using a special version of Easypop 1.8 which allows analysis of allele frequency changes over time (Balloux 2001). First, we simulated a large panmictic population ($N_e = 10{,}000$) for 1,000 generations to generate 100 independent microsatellite loci (maximum number of alleles = 14). The microsatellite mutation rate was set to 2.5×10^{-4} according to the MSVAR outcome (see below). This was in between the mutation rate used by Garza and Williamson (5.0×10^{-4}; Garza and Williamson 2001) and the average microsatellite mutation rate in zebrafish (1.5×10^{-4}; Shimoda et al. 1999). Simulations assumed a TPM comprising 90% single step and 10% multi-step mutations, which was chosen in order to be directly comparable with the simulations of Garza and Williamson (2001). During the second phase of simulations, the single panmictic population was split into two groups containing a single large population ($N_e = 5{,}000$) and 100 small populations ($N_e = 50$, referring to the minimal population size for short term persistence). Migration was allowed between the large and small populations, but not among the small populations. Six different migration levels (*m*) were tested: 0, 0.001, 0.01, 0.02, 0.05 and 0.1. The second phase of simulations was run for 3,000 generations and data were collected at generation 0, 10, 50, 100, 200, 350, 500, 1000, 2000 and 3000 to calculate the *M* ratio as described earlier.

Estimation of effective population size

Compared to the genetic bottleneck tests described above, estimation of effective population size (N_e) could provide complementary information about the bottleneck process. In this study, the N_e was estimated from a single temporal sample using an approximate Bayesian computation framework (Tallmon et al. 2004) implemented in the program ONeSAMP 1.1 (Koyuk et al. 2008; Tallmon et al. 2008). In contrast to other single-sample methods that are based on one parameter estimation, the ONeSAMP N_e estimator combines eight summary statistics: the *M* ratio (Garza and Williamson 2001), the difference of the natural logarithms of variance in allele length and heterozygosity (King et al. 2000), expected heterozygosity, number of alleles per locus, Wright's F_{IS}, the mean and variance of multilocus homozygosity and the square of the correlation of alleles at different loci based on linkage disequilibrium (Hill 1981). As a result, ONeSAMP is expected to provide more accurate and precise N_e estimates compared to alternative single-sample methods relying on only one genetic parameter (Tallmon et al. 2008). ONeSAMP 1.1 requires the prior setting of lower and upper bounds for N_e. To limit the lower and upper bounds of N_e, a prior minimum N_e was set to two (the lowest value) and a prior maximum N_e was set to 500 according to a preliminary survey using the linkage disequilibrium method (Hill 1981) implemented in NeEstimator 1.3 (Peel et al. 2004). We also assessed the effect of maximum prior N_e 1000 in some populations but the N_e estimates were highly similar compared to maximum prior 500 (data not shown).

To evaluate the effect of migration to the N_e estimates we utilized the simulated datasets that were generated earlier to describe the effect of gene flow on the dynamics of *M* ratio (see above). N_e was estimated in simulated populations at the 50th and 2000th generations with six migration levels ($m = 0$, 0.001, 0.01, 0.02, 0.05 and 0.1; each comprising 10 replicates) using the linkage disequilibrium method (Hill 1981) implemented in NeEstimator 1.3 (Peel et al. 2004). The linkage disequilibrium method was used because estimation of N_e using the approximate Bayesian computation framework is very time-consuming with a large number of simulated datasets. However, we estimated the N_e using both methods in a subset of datasets for comparative purposes and the point estimates were highly similar (data not shown). The 50th and 2000th generations were chosen to represent populations in non-equilibrium and equilibrium phases, respectively.

Results

Microsatellite diversity, Hardy–Weinberg and linkage equilibrium

The average allelic richness varied from 1.7 (Les) to 4.77 (Ram) while the expected heterozygosity ranged from 0.24 (Les) to 0.64 (Hol). The proportion of polymorphic loci

ranged from 0.5 (Kai) to one (in Hol, Kem, Kit, Kru, Pur, Skj and Tor; Table 1). Deviation from Hardy–Weinberg was observed ($P < 0.05$) in five populations: Rau ($P = 0.004$), Pur ($P = 0.006$), Vat ($P = 0.017$), Ege ($P = 0.035$) and KasP ($P = 0.044$); none of these remained significant after the sequential Bonferroni correction (cutoff P-value = 0.0017 at $k = 30$). Significant deviation from linkage disequilibrium was detected in 143 cases ($P < 0.05$) out of 1893 tests. The number of locus pairs showing linkage disequilibrium per population ranged from 0 to 26 (in Kit), while each locus pair exhibited significant linkage disequilibrium only in a small number of populations (1–6). After the sequential Bonferroni correction, however, only three of these remained significant (Bfro12–Cocl23 in Juu, Bfro13-Bfro7 in Esa, Bfro13–Bfro11 in Kit, all associated $P < 2.6 \times 10^{-5}$). For the subsequent analyses we therefore assumed that all studied loci were physically unlinked and independent.

M ratio test

The observed *M* ratio was lower than the commonly used bottleneck threshold (0.68; Garza and Williamson 2001) in all grayling populations, ranging from 0.44 (Ege) to 0.66 (KasL). Nearly half of the populations (14 of 34, 41%) had an upper 95% CI below the threshold, indicating rather severe bottlenecks (Fig. 2). The calculated critical M_c values from setting 1 (90% SMM) were all higher (range 0.62–0.73) than the observed *M* ratio estimates for analyzed grayling populations (Fig. 2). As expected, the calculated critical M_c values from setting 2 (80% SMM) were lower (range 0.54–0.68) than for setting 1. However, even with 80% SMM model, 30 populations out of 34 still exhibited *M* ratios lower than the calculated critical bottleneck threshold (Fig. 2). This indicates that even relatively strong deviations from SMM cannot explain the extremely low *M* ratio estimates in the majority of the studied grayling populations.

Wilcoxon's test and mode-shift test

Using pure stepwise mutation model (SMM), the Wilcoxon's test detected a recent bottleneck footprint only in one population out of 34 (Rau, $P = 0.039$, uncorrected for multiple test). Under the more realistic (Di Rienzo et al. 1994) two-phase mutation model (TPM; 95%SMM and 5%IAM), the Wilcoxon's test revealed recent bottleneck footprints in four populations—Rau, Iso, Kai and Skj (uncorrected $P = 0.026$, 0.034, 0.039 and 0.040, respectively; Table 1). However after sequential Bonferroni correction, none of the populations showed significant heterozygote excess with the Wilcoxon's test. The mode-shift test revealed the distortion from L-shaped allele

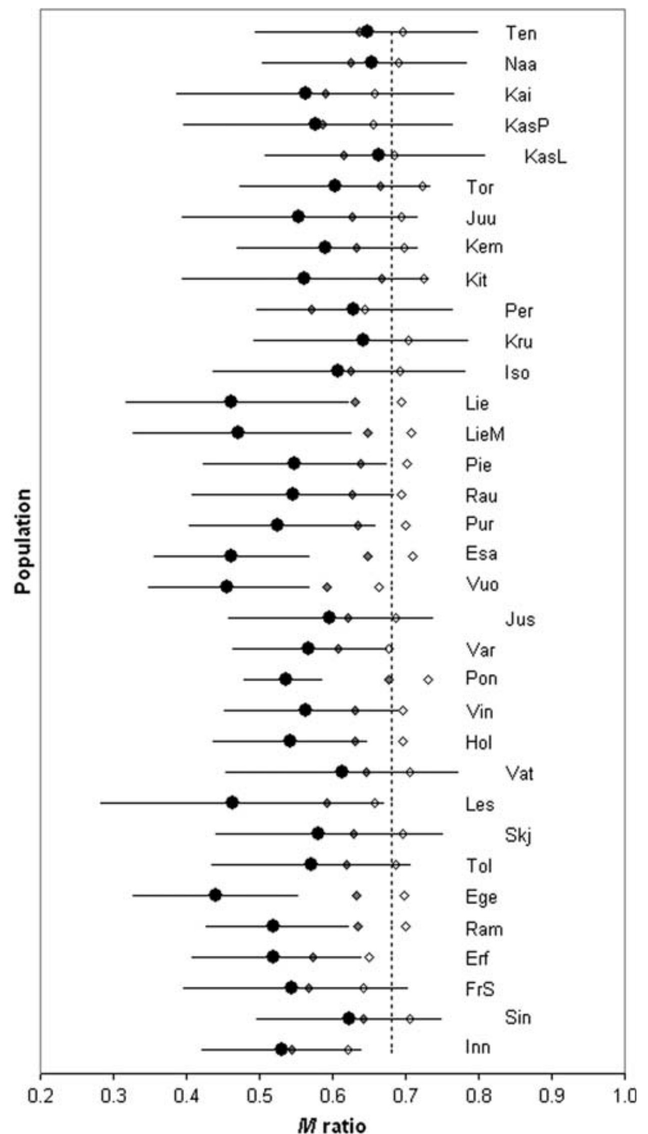

Fig. 2 Calculated *M* ratio in European grayling populations (95% CI indicated as horizontal lines). The broken vertical line indicates the common threshold for genetic bottleneck suggested by Garza and Williamson (2001). Open and gray diamonds denote calculated critical threshold for *M* ratio (M_c) corresponding to 90% and 80% single-step mutations, respectively

frequency distribution in four populations: Kai, Les, Lie and Rau. However, we were not able to apply multiple correction for mode-shift test, as BOTTLENECK 1.2.02 (Piry et al. 1999) does not provide population-specific significance values associated with the distortion from the expected allele frequency distribution.

Dating of demographic change

Coalescent analyses using a hierarchical Bayesian model and assuming exponential population growth/decline indicated that all 34 grayling populations exhibit signals of

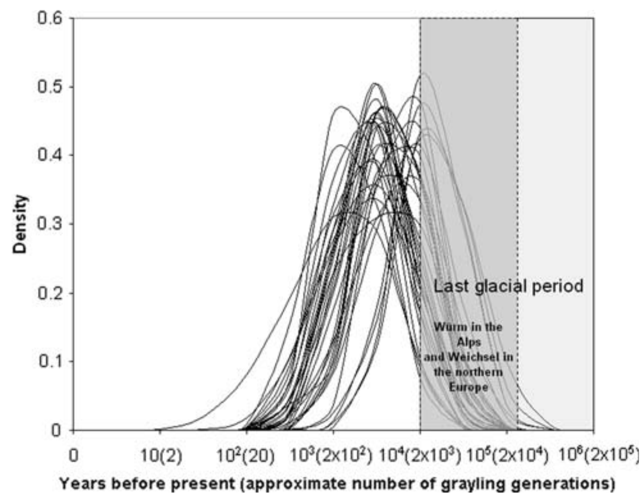

Fig. 3 Estimated time since the start of population decline (posterior distribution) in 34 grayling populations across Europe using an exponential model of MSVAR (Beaumont 2004). The gray shaded area corresponds to the last glacial period and its last sub-period (Würm and Weichsel period)

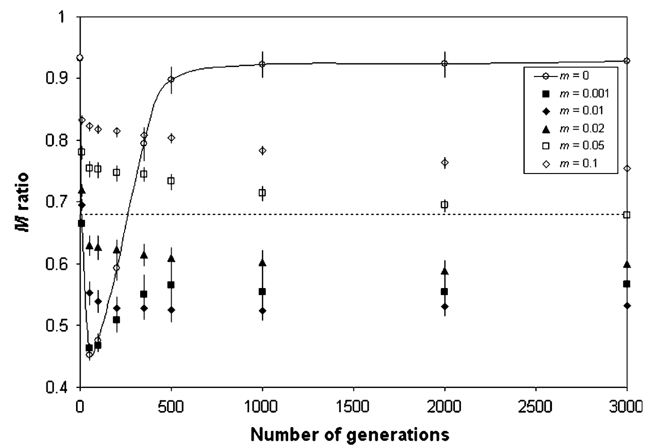

Fig. 4 The dynamics of the M ratio (means and 95% CI) in the presence of migration (m) during the permanent bottleneck scenario. The uppermost circle on the left-side of the figure corresponds to the initial M ratio before the population reduction from 10,000 to 50. A broken horizontal line indicates the critical bottleneck threshold at 0.68 according to Garza and Williamson (2001)

dramatic size reduction. Current population sizes were estimated to represent only a small fraction compared to historical sizes (0.03–1.2%, details not shown). The derived mean mutation rate across loci was similar among populations at $2.4–2.6 \times 10^{-4}$ per generation. The estimated start of population decrease was dated back to 1,000–10,000 years (Fig. 3). The simulations based on a linear model indicated similar population size reductions and mutation rates but the start of population decrease was dated back to 32,000 to 100,000 years (data not shown).

The dynamics of M ratio in the presence of migration

As expected, the M ratio of a large simulated population was high (M ratio = 0.927, ranging from 0.912 to 0.945) indicating that the population was at mutation-drift equilibrium. However when population size was reduced to 50, the estimated M ratio dropped rapidly (Fig. 4). When small populations were kept isolated, the M ratio recovered after ca. 300 generations, similar to what was observed by Garza and Williamson (2001). However, when looking the number of alleles present in the population after 300 generations, it was clear that the recovery of M ratio was caused by the fixation of alleles, as essentially no variability was left in the population. Very different M ratio dynamics was observed when the migration rate (m) was kept between 0.001 and 0.02 as the M ratio remained below 0.68 up to 3,000 generations (Fig. 4). When the migration level was higher (m = 0.05–0.1), the M ratio did not drop below 0.68 even after the population reduction, as gene flow efficiently counteracts the loss of alleles by genetic drift.

Effective population size

Estimated effective population sizes for all grayling populations were very small, ranging from 16.5 (Kai, 95% credible limits CL = 12.7–29.2) to 71.4 (Tor, 95% CL = 52.9–138.4), with an average N_e just 25.7. Notably, even the upper 95% CL were smaller than 50 for all populations, except Pon, Ten and Tor (Fig. 5). The more traditional linkage disequilibrium method (Hill 1981) also provided low N_e estimates (mean across populations = 89.4), but considerably wider confidence intervals (95% CI ranging from 8.0 to infinity).

When N_e was estimated from the simulated data (true N_e = 50) with different levels of migration, the N_e estimates were slightly biased upwards in case of no migration (N_e = 55.9; 95% CI = 53.0–58.6) and very low migration (m = 0.001; N_e = 57.5; 95% CI = 46.3–66.0). In contrast, the N_e estimates were slightly biased downwards in case of higher migration (m = 0.01, N_e = 26.4, 95% CI = 16.5–36.7; m = 0.02, N_e = 26.3, 95% CI = 19.6–34.3). Nevertheless, even when the simulated populations did not correspond to totally closed systems, the N_e estimates based on linkage disequilibrium were relatively close to the true N_e. Congruent to the empirical data, the point N_e estimates derived from ONeSAMP and NeEstimator were very similar.

Discussion

We identified a strong genetic bottleneck footprint in all 34 analyzed grayling populations over continental Europe using the M ratio test while only a small subset of populations showed signatures of population decline using

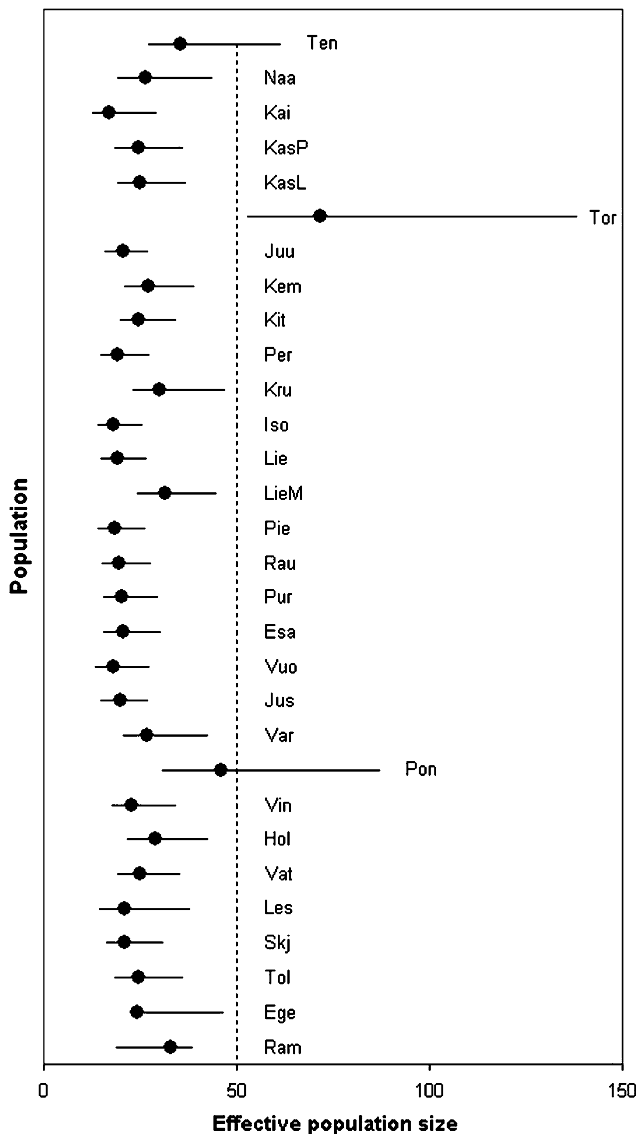

Fig. 5 Estimated effective population sizes in European grayling populations (95% CL indicated as horizontal lines). The broken vertical line indicates $N_e = 50$

mode-shift and heterozygote excess tests. Using coalescent analyses, the beginning of population reduction was dated back 1,000–10,000 years (ca. 200–2,000 generations assuming an exponential model) and current population sizes were estimated to represent only 0.03–1.2% of historical sizes. In the following sections, we discuss these results in the light of assumptions of the bottleneck tests, persistence of bottleneck footprints, phylogeographic history of European grayling and the implications of our findings for conservation.

The influence of gene flow on the *M* ratio

When a population remains relatively small and isolated, the *M* ratio test is expected to detect a bottleneck that dates back 100–300 generations at most (Garza and Williamson 2001). This would imply that the low *M* ratio observed in all 34 grayling populations might be caused by independent genetic bottlenecks that occurred maximally 500–1500 years ago assuming a generation time of 5 years (Haugen and Vøllestad 2001). However, coalescent analyses assuming an exponential model of population growth/decline dated the beginning of population reduction back to 1,000–10,000 years (ca. 200–2,000 generations) while the estimated start of population decrease assuming a linear model was dated back even further (32,000 to 100,000 years, ca. 6,000–20,000 generations). We subsequently investigated this inconsistency in timing of population reduction by using forward population genetic simulations to evaluate the long-term dynamics of *M* ratio in the face of gene flow. In contrast to the isolated population scenario used by Garza and Williamson (2001) we observed that low *M* ratio can persist within a small population much longer than previously anticipated when a relatively low level of gene flow is present. Specifically, low-medium levels of migration were sufficient to avoid fixation of alleles in a small population, but insufficient to sustain or recover all original alleles and therefore the *M* ratio did not return to its initial level even after 3,000 generations. Hence, based on coalescent and forward population genetic simulations, we suggest that the low *M* ratio in European grayling is more consistent with the common historical genetic bottleneck scenario related to the last glacial period and subsequent post-glacial recolonization. This is a plausible scenario for explaining the low *M* ratios in European grayling but is also an important point to consider in studies of other species where migration rates are likely to be low, but non-zero, such as other freshwater fishes and other taxa with high breeding site fidelity and/or poor dispersal ability. In contrast, our results also suggest that low *M* ratios are unlikely to be observed in species with high gene flow levels, even following a dramatic reduction in population size. This highlights the fact that, negative results do not necessarily imply that population size crashes have not occurred (Busch et al. 2007).

An alternative process, which, at least in theory, can result in low *M* ratio, is related to a violation of the stepwise mutation model in the analyzed microsatellite loci (Guinand and Scribner 2003; Williamson-Natesan 2005). However, even when we assumed relatively strong deviation (80% SMM and 20% IAM) from pure SMM, bottleneck signatures were still observable in 30 out of 34 populations. Taken together, our results indicate that gene flow can have a strong influence on the persistence of bottleneck footprints and the recovery of *M* ratio to its initial value can take much longer than previously anticipated. These findings are consistent with empirical tiger salamander (*Ambystoma*

tigrinum) data by Spear et al. (2006) who demonstrated that the *M* ratio most likely has a slower recovering rate than heterozygote excess or allele frequency shift test. Our results are also in accordance with Williamson-Natesan (2005) who demonstrated that violations of the bottleneck test assumptions can represent major sources of error when screening for genetic bottlenecks. Therefore, assessing the appropriateness of the model assumptions in bottleneck detection methods should remain an important focus in conservation genetics research.

Glacial history and long term demographic changes in European grayling

European grayling throughout Europe consists at least three major highly diverged mitochondrial lineages originating from different glacial refugia for which the time of lineage separation is estimated to be approximately 400,000 years (Koskinen et al. 2000; Gum et al. 2005). This divergence clearly predates the late Pleistocene period (130,000–10,000 years) implying that European grayling survived dramatic temperature oscillations in distinct refugia (see review in Gum et al. 2009). Our study included 34 populations originating from the three lineages and the coalescent analyses using an hierarchical Bayesian model suggested that all grayling populations have experienced dramatic size reductions that date back 1,000–10,000 or 32,000–100,000 years, depending whether an exponential or linear population growth/decline model was used. As far as we are aware of, it is not possible to determine which model better fits the empirical data so we focus our discussion on the results based on the exponential model as it is most widely applied (Storz and Beaumont 2002; Storz et al. 2002). The use of the alternative linear model, however, does not change the main conclusions as the estimated time of separation of three mitochondrial lineages based on mitochondrial DNA predates the signal of population decline derived from microsatellite data regardless of the model applied. This suggests that population size reduction in grayling probably occurred independently in the three lineages after the last glacial period and current population sizes represent only a small fraction compared to historical sizes (0.03–1.2%). Similarly, Meldgaard et al. (2003) used the same methodology and estimated that the population declines of grayling in Denmark began ca. 7,500 years ago and the contemporary populations were only 2–5–3.3% of historic sizes. The signatures of population decline in European grayling may be therefore related to colonization processes after the last glacial period. These observations are also congruent with Arctic grayling (*Thymallus arcticus*), the sister species of European grayling, for which the contemporary populations have been estimated to represent less than 1% of historical sizes (Stamford and Taylor 2005). It is also in concordance with studies of several other fish species and fauna in the northern hemisphere where profound effects of founder events during the post-glacial recolonization have been found (Bernatchez and Wilson 1998; Hewitt 1999; Taylor and McPhail 2000; Kontula and Vainola 2001; Costello et al. 2003).

Recent genetic bottlenecks in European grayling

In contrast to the *M* ratio test, only a subset of populations (6 populations out of 34) exhibited recent bottleneck footprints using the heterozygosity excess and mode-shift test. This is in accordance with the earlier studies that have reported only a single recent genetic bottleneck signature in European grayling (Gum et al. 2003). In the present study, only two populations were identified (Kai and Rau) using both the heterozygosity excess and mode-shift test. This was not unexpected, however, as both the sensitivity and power of bottleneck tests differ from each other. For example, the mode-shift test is expected to be relatively insensitive to the assumptions regarding the mutation model and enables detection of rather recent reductions in population size that occurred maximum a few dozen generations ago (Cornuet and Luikart 1996). However, the mode-shift test also appears to have rather limited power to detect sharp population declines (Williamson-Natesan 2005). Hence, based on the analyzed data, it is likely that the four grayling populations (Kai, Les, Lie and Rau) that exhibited allele frequency shifts have indeed suffered recent population decline. Interestingly, the population from Norway (Les) also exhibited extremely low level of genetic variability ($Ar = 1.81$; $H_E = 0.24$), which is in accordance with the demographic history of the population as it is known to be established by stocking of small number of individuals in 1880 (Koskinen et al. 2002a). Assuming the average generation time of 5 years and the Norwegian sample (Les) was collected in 1999 (Koskinen et al. 2002b), this would transform to approximately 24 generations which is close to the upper detection limit for mode-shift test. However, an even lower level of diversity ($Ar = 1.56$, $H_E = 0.20$) and an allele frequency shift was observed in a grayling population (Kai) from northern Finland.

Compared to the mode-shift test, heterozygosity excess is expected to be detectable in a population for a longer period of time, depending on the effective population size (Cornuet and Luikart 1996) before the population reaches a new mutation-drift equilibrium (Maruyama and Fuerst 1985). In addition, the heterozygosity excess test is expected to detect sharp population declines more often than the mode-shift test but at the same time it is also more sensitive to violations of the mutation model (Williamson-

Natesan 2005). We identified four populations (Iso, Kai, Rau and Skj) that showed weak but significant heterozygosity excess (TPM; $P < 0.05$) and two of these (Kai and Rau) also exhibited bottleneck signals in the mode-shift test. However, no cases of heterozygosity excess remained significant after correction for multiple tests. That said, we argue that making a type II error i.e. failing to detect bottleneck when it is present can be more harmful in a conservation context than claiming genetic bottleneck when there is none. Hence, six grayling populations (Iso, Kai, Les, Lie, Rau and Skj) might have suffered sharp relatively recent population declines and thus, from a conservation perspective, warrant future monitoring. Our results also highlight the utility of using alternative statistical tests for detection and timing of genetic bottlenecks (Spear et al. 2006).

Effective population size and population subdivision in European grayling

Based on a single time-point analysis using approximate Bayesian computation methodology, all grayling populations exhibited very small effective population sizes with the majority of N_e estimates below 50, a commonly used threshold for short term population persistence. However, there are several complicating factors that can bias N_e estimates (Palstra and Ruzzante 2008) and below, we discuss some of the critical assumptions related to estimation of N_e from a single time-point. First, compared to the temporal change method (Waples 1989), the period accounted in estimating N_e using a single population sample is worth consideration. In principle, ONeSAMP combines eight different summary statistics (including *M* ratio) for estimation of N_e but it relies largely on the linkage disequilibrium among loci (Tallmon DA, personal communication). As a result, our N_e estimates probably reflect more recent N_e rather than historical N_e. The second critical concern is related to the reliability of N_e estimates using rather small sample sizes. For example, England et al. (2006) demonstrated that the N_e estimate is likely to be biased downwards if the sample size is smaller than the estimated N_e. In this study, the sample sizes were larger than the estimated N_e in 26 cases out of 30, indicating that these N_e estimates are unlikely biased towards smaller values. However, it is also worth noting that the two populations with relatively large sample sizes had the large N_e estimates (Pon $n = 112$, N_e estimate $= 45.6$; Tor $n = 63$, N_e estimate $= 71.4$) and clearly, analyses of more individuals would be useful for proper evaluation of the effect of sample size using an approximate Bayesian computation methodology. Third, N_e estimates can be biased if the effect of migration is not taken into account (Palstra and Ruzzante 2008). For example, it has been shown that gene flow can bias N_e estimates both downwards and upwards depending on the type of migration when using a temporal method for N_e estimation (Palstra and Ruzzante 2008). Unfortunately, currently available methods for estimating N_e based on single population sample do not account for the effects of gene flow (but see Vitalis and Couvet 2001). To evaluate the potential bias in our N_e estimates related to gene flow, we estimated effective population sizes from simulated populations experiencing different levels of migration. However, migration did not strongly affect N_e estimates in these simulated populations. Nevertheless, it is possible that undetected admixture and population substructure can seriously bias N_e estimates, especially when the populations are highly differentiated.

The very small effective population sizes observed in grayling are in accordance with our previous genetic analyses (Swatdipong et al. 2009) indicating that genetic drift has played a major role shaping the diversity and population structure in European grayling. However, these findings are in contrast with the current conservation status of many of the populations. Although grayling populations are declining in many parts of Europe (Uiblein et al. 2001; Duftner et al. 2005), a survey of the status of 253 grayling populations in Finland indicated that only 16% of populations were considered as declining, vulnerable or endangered (Kaukoranta et al. 2000) and the annual recreational angling catch in Finland is around 180 tonnes (Nylander 2004). Why then, are N_e estimates of grayling populations invariably low? We suggest that the high level of site fidelity observed in grayling results in extremely localized sub-populations each of which may have a relatively small effective population size, even when the N_e of an entire watershed can be much larger. Thus, if genetic analyses are conducted on samples collected from a single watershed location, as is often the case in broad geographic studies, then N_e estimates will most likely reflect the local sub-population rather than that of the overall water system. This notion is supported by earlier studies which have demonstrated very low levels of within population genetic diversity in grayling (Koskinen et al. 2002c; Susnik et al. 2004; Gum et al. 2006) compared to other salmonid fishes (Vasemägi et al. 2005; Gross et al. 2007; Fave and Turgeon 2008; Lehtonen et al. 2009), as well as high levels of genetic divergence between grayling populations separated by just tens of kilometers (Koskinen et al. 2001). Nevertheless, gene flow most likely plays a crucial role counteracting the negative effects of genetic drift and loss of variation of grayling populations in the long run. In the future, more detailed studies examining e.g. the temporal stability of allele frequencies, population structure within water systems as well as investigations of homing behavior in European grayling are warranted in order to shed more light on these issues.

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