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RESEARCH ARTICLE

Substantial genetic structure among stocked and native populations of the European grayling (*Thymallus thymallus*, Salmonidae) in the United Kingdom

Nick Dawnay · Louise Dawnay · Roger N. Hughes · Richard Cove · Martin I. Taylor

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Abstract While currently in a state of recovery in the United Kingdom (UK), the grayling (*Thymallus thymallus*) remains of conservation interest due to its historical decline, socio-economic value and the potential impact of hatchery-reared stock fish on the genetic structure and diversity of wild populations. However, little is known about the levels and distribution of genetic diversity among UK grayling populations. To this end, 27 UK populations of grayling were genotyped across 10 microsatellite loci and sequenced at the mtDNA D-Loop. All populations clustered into four higher-level groups: Northern England, Southern England, Wales, and group consisting of a mixture of native and introduced populations. Ten populations showed evidence of bottleneck or founder effects, and the effective population size (Ne) was low in all populations. In most cases, historical stocking records agreed with the genetic relationships revealed in the study. A D-Loop haplotype network supported the groupings observed in the nuclear data, while phylogenetic inference places the UK populations amongst Central European samples. The combined datasets demonstrate that many of the UK populations can be treated as separate Management Units and we recommend that to preserve population specific genetic diversity, that stocking should be an intervention of last resort. However, if stocking is deemed essential, brood stock should originate from the river to be stocked.

Keywords Conservation · Population structure · Genetic · Bottleneck · Stocking · Ne · *Thymallus*

N. Dawnay · L. Dawnay · R. N. Hughes · M. I. Taylor (✉)
Molecular Ecology and Fisheries Genetics Laboratory, School of Biological Sciences, Bangor University, ECW Building, Bangor, Gwynedd LL57 2UW, UK
e-mail: m.taylor@bangor.ac.uk

R. Cove
Environment Agency, Chester Road Buckley, Flintshire CH7 3AJ, UK

Introduction

European grayling (*Thymallus thymallus*) are widely distributed over Northern Europe and Russia, extending from Wales in the West to the Ural mountains in the East and have undergone significant declines in numbers in many populations due to environmental degradation and over-exploitation (Gum et al. 2009). Conservation assessments made during the 1980s concluded that the species was endangered in the UK and threatened within Europe (Lelek 1984) and in an effort to conserve the species, stocking has been implemented in a number of European countries (Ibbotson et al. 2001; Sušnik et al. 2004; Gum et al. 2006). The current global IUCN classification for the European grayling is "lower risk, least concern" (Freyhof and Kottelat 2008). However, continuing habitat degradation and over-fishing at the local scale mean that the active conservation of grayling remains of pressing importance in Europe (e.g. Meldgaard et al. 2003; Bergerot et al. 2008; Gum et al. 2009).

Genetic investigations of European grayling have primarily focussed on contemporary population connectivity (Gross et al. 2001; Koskinen et al. 2002a; Gum et al. 2005) and phylogeographic history (Koskinen et al. 2000; Weiss et al. 2002; Koskinen et al. 2002b), although more recent investigations have focused on genetic impacts of stocking (Duftner et al. 2005; Duchesne and Turgeon 2009). Such studies have demonstrated that many continental European

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populations display marked genetic differences at both nuclear and mitochondrial loci and a number of mitochondrial lineages have been identified (Koskinen et al. 2000; Gum et al. 2005). However, in addition to the high levels of genetic differentiation among many grayling populations, evidence of low effective populations sizes and population bottlenecks have recently been identified in 34 European populations (Swatdipong et al. (2010), suggesting that genetic drift is likely to have played a powerful role in determining levels of genetic variation among grayling populations.

Grayling are native to many parts of mainland British Isles (although they are absent from Ireland) but their range has been significantly increased through stocking (Wilson 1966; Wheeler 1977; Ibbotson et al. 2001). Native populations are thought to be restricted to the Rivers Ouse, Trent, Hampshire Avon and possibly the rivers Severn, Wye, Ribble and Welsh Dee and their tributaries (Gardiner 1989; Ibbotson et al. 2001). Introduced populations can now be found in Scotland (where none are native) and in a large number of rivers not previously thought to be naturally inhabited by grayling (Ibbotson et al. 2001). Stocking of both native and introduced populations continues without knowledge of the genetic relationships among the source and recipient populations, and so could have deleterious effects on stocked populations. Hence, it is important to assess genetic differentiation among and genetic variation within populations and to define and implement measures to safeguard genetic variability (e.g. Taberlet and Bouvet 1994; Baker et al. 1998; Dawnay et al. 2008).

Genetic data for UK grayling are currently limited to two southern English populations that were included in a larger study investigating genetic diversity and phylogenetic relationships of European populations (Gum et al. 2005). A high level of population structuring was observed between these two populations at microsatellite loci (Gum et al. 2005), suggesting UK populations show similar levels of genetic structure populations elsewhere in Europe. These data were also used to infer postglacial colonisation from central Europe via the North Sea River prior to the isolation of Britain from continental Europe (Gum et al. 2005). Elucidating the genetic relationships among UK grayling populations and within a wider European context is essential for planning and implementing conservation programs within the UK. In the present study we use 10 nuclear microsatellite markers and partial mtDNA control region (or D-Loop) sequences to quantify the levels of genetic variation within and among 27 UK populations of grayling. These data are used to investigate the origins of the UK grayling fauna (both introduced and native populations), levels of genetic diversity within and genetic structuring among UK grayling populations.

Materials and methods

Sample collection and DNA extraction

Population samples were collected from 27 localities were sampled that covered the majority of the native UK populations and a representative number of introduced populations providing (Table 1; Fig. 1). Adult grayling samples were collected using rod and line from between 1 and 5 km stretches of river at each sampling site during spring and summer 2008. Fin-clips were taken from 1183 individual fish and preserved in 95% ethanol (Table 1; Fig. 1). Based on previous research by Ibbotson et al. (2001), census information from The Grayling Society and historical reports, sample sites were categorised as post-glacial 'native' populations, recent 'introduced' populations or 'native-stocked' populations (Table 1). DNA was extracted using a salt-precipitation technique modified from Aljanabi and Martinez (1999) and resuspended in 100 µl of H_2O. Negative controls were used throughout extraction.

Microsatellite analysis

Ten previously isolated microsatellite loci were selected based on the observed level of polymorphism in other studies, their adherence to Hardy–Weinberg equilibrium and the ability to be multiplexed (Table 2). The forward primer of each locus was 'tailed' to allow annealing of a fluorescently labelled oligonucleotide following the method of Schuelke (2000) (Table 2). Loci were amplified in 10 µl reactions in two multiplexes using Qiagen Multiplex PCR kit (Qiagen, USA) following manufacturer's instructions. PCR (Saiki et al. 1985) amplification was performed on a MJ Research PTC-200 thermocycler using the following cycling parameters: 15 min denaturation step at 95°C; 35 cycles of 94°C for 30 s, 55°C for 90 s, and 72°C for 90 s; 30 min elongation step at 60°C. PCR products were resolved on an ABI 3130*xl* genetic analyser (Applied Biosystems) using Genescan™ 500 LIZ® internal size standard (Applied Biosystems). Positive controls were included in each run to allow for calibration between runs. Fragments were sized using GENEMAPPER v4.0 software (Applied Biosystems).

Allele frequencies, observed and unbiased expected heterozygosities under Hardy–Weinberg expectations were calculated using GeneClass2 (Piry et al. 2004). Departures from Hardy–Weinberg equilibrium (HWE) were tested for significance using the probability test implemented within GENEPOP 3.3d (Raymond and Rousset 1995). Significance levels were determined using the Markov chain method (dememorisation number = 10000, batches = 100, iterations = 10000). Genotypes at all pairs of loci were tested for linkage disequilibrium using the exact test implemented in

Table 1 Sample sites and associated map codes, population classification and associated citations, number of grayling genotyped (N), expected heterozygosity (H_E), observed heterozygosity (H_O), loci which deviated from Hardy–Weinberg expectations (HWE), mean allelic richness (N_A) and inbreeding coefficient (Fis) and effective population size (Ne) and 95% confidence intervals measured from up to 10 variable microsatellite loci

River	Pop code	EA region[a]	Population classification	N	H_E	H_O	HWE	N_A	Fis	Ne	Ne 95% CI
Wharfe	WRF	NE	Native stocked[b,c]	21	0.46	0.42	A	3.35	0.09*	33.4	26.8–89.1
Yorkshire Derwent	YKD	NE	Native[b]	51	0.26	0.27		2.18	−0.07	53.4	35.9–151.3
Driffield West Beck	DWB	NE	Introduced[b]	19	0.35	0.38		2.39	−0.08	20.1	17.6–36.4
Ure	URE	NE	Native[b]	58	0.35	0.32	E	2.93	0.09*	62.5	52.5–261
Aire	AIR	NE	Native stocked[d,e,b]	39	0.45	0.44		2.98	0.02	63.9	40.6–139.2
Pickering Beck	PKB	NE	Native[b]	40	0.37	0.39		2.63	−0.05	47.2	42.4–164.9
Kennet	KEN	T	Introduced[b]	49	0.29	0.27	C*	2.00	0.08	32.4	30.1–143.8
Test	TST	S	Introduced[b,c]	53	0.44	0.48		2.57	−0.07	40.3	32–66.8
Itchen	ITH	S	Introduced[c]	50	0.39	0.38		2.52	0.02	86.6	60.3–296.9
Dorset Frome	DRF	SW	Introduced[f]	53	0.36	0.37		2.34	−0.05	69.8	51–187.3
Hampshire Avon	HAV	SW	Native stocked[b]	58	0.42	0.45		2.49	−0.07	32.5	23–55.4
WylyeA	WLA	SW	Native[f]	48	0.34	0.32		2.18	0.05	33.5	21.8–78.7
WylyeB	WLB	SW	Native[f]	51	0.40	0.40		2.22	−0.01	39.7	30.6–83.1
Wye	WYE	W	Native[b,c]	55	0.40	0.40	C	2.95	0.00	121	81.6–372.7
Dee	DEE	W	Native[b,c]	52	0.54	0.51	E	3.47	0.04	43.2	31–75.4
Irfon	IRF	W	Native[f]	24	0.37	0.40		2.87	−0.08	29.6	28.4–69.2
Derbyshire Derwent	DBD	M	Native stocked[d,b]	39	0.42	0.45		2.66	−0.07	36.9	30.1–62.8
Dove	DOV	M	Native stocked[e,b]	50	0.35	0.32	A, H	2.63	0.09	64.4	36.7–170.5
Severn	SEV	M	Native[b,c]	39	0.42	0.41		2.77	0.03	40.8	36–128.2
Eden	EDN	NW	Introduced[b,g,c]	45	0.40	0.38	A, D	2.51	0.04	48.7	30.4–130.6
Ribble	RIB	NW	Native stocked[b,g]	42	0.37	0.37	J	2.47	−0.02	30.7	26.4–80.9
Earn	ERN	SC	Introduced[b,h]	49	0.25	0.28		1.80	−0.12*	34.6	25.5–74.6
Annan	ANN	SC	Introduced[b,h]	41	0.12	0.09	J	1.43	0.24*	25.5	14.2–61.7
Clyde	CLD	SC	Introduced[b,h]	64	0.39	0.37	G	2.26	0.05	68.6	47.9–209.8
Gryffe	GRF	SC	Introduced[b,h]	25	0.43	0.45		2.61	−0.06	12	10–17.8
Tweed	TWE	SC	Introduced[b,h]	21	0.39	0.34	C	2.58	0.13	22.1	16.6–48.8
South Calder	SCA	SC	Introduced[b,h]	47	0.17	0.18	C*	1.60	−0.09	15.1	11.3–27.7

A = Ogo2, B = BFRO005, C = BFRO018, D = BFRO011, E = ONE2, F = BFRO015, G = BFRO010, H = BFRO016, I = BFRO016, J = BFRO012

SW South West, *S* Southern, *T* Thames, *W* Wales, *M* Midlands, *A* Anglia, *NE* North East, *NW* North West, *SC* Scotland

* Indicates significant value after Benjamini and Hochberg correction

[a] Environment Agency monitoring regions, [b] Wilson (1963), [c] Ibbotson et al. (2001), [d] Roger Walker (pers.com), [e] http1, [f] Inferred, [g] Wilson (1966), [h] http2

GENEPOP 3.1c with significance levels determined by the Markov chain method (dememorisation number = 10000, batches = 100, iterations = 10000). A test to identify historical genetic bottlenecks or 'founder effects' was performed using the software Bottleneck (Cornuet and Luikart 1996; Piry et al. 1999). Populations that have experienced a rapid decline in size are expected to have excess heterozygosity relative to that expected under mutation-drift equilibrium (Heq). Heq was calculated using the two-phase mutation model (TPM) (Piry et al. 1999) with a probability of 95% for single step mutations (SMM) and 5% multi-step mutations (Di Renzo et al. 1994), the variance for mutation size was set to 12 (Piry et al. 1999). A bottleneck was detected by the occurrence of a mode-shift and/or a significant excess heterozygosity detected by a Wilcoxon sign-rank test. A Kruskall-Wallis non-parametric test was performed in SPSS 14 (SPSS Inc, Chicago, IL) to test for significant differences between expected heterozygosity and number of alleles between native, native-stocked and introduced population groups. Effective population size (Ne) (using a single point sample for each population) was estimated using an approximate Bayesian framework implemented within ONeSAMP (Tallmon et al. 2008; Koyuk et al. 2008) which combines eight summary statistics to estimate Ne (Tallmon et al. 2008). All Ne estimates were performed three times per population using a lower bound of

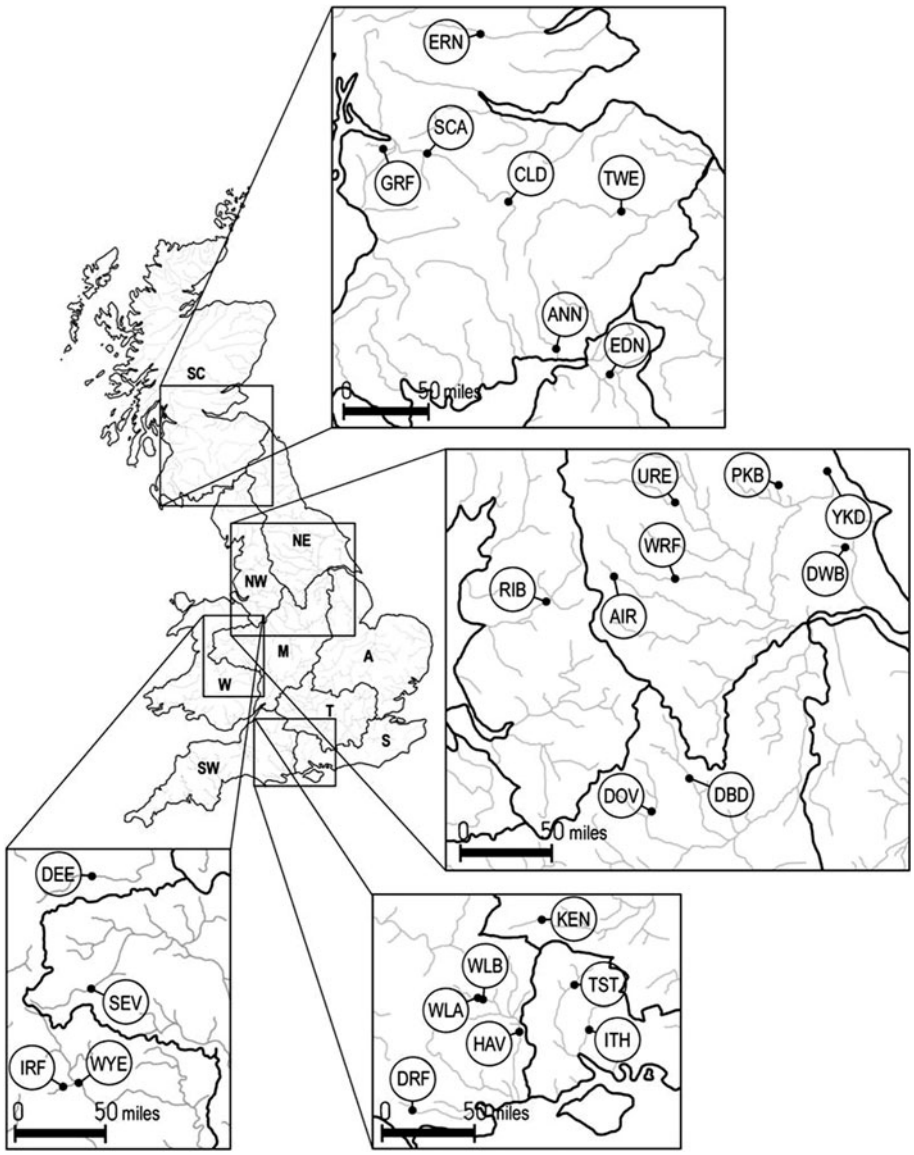

Fig. 1 UK grayling population sites with three letter sample site codes. Code key provided in Table 1. Regions shown on complete map refer to Environment Agency monitoring regions with *SW* South West, *S* Southern, *T* Thames, *W* Wales, *M* Midlands, *A* Anglian, *NE* North East, *NW* North West, *SC* Scotland

4 and an upper bound of 1000. The median estimates from the three runs are reported.

Population differentiation was estimated using Weir and Cockerham's (1984) F-statistic θ and G-statistics were calculated with 2000 permutations and 2000 bootstraps to estimate statistical significance of differentiation in FSTAT 2.9.3 (Goudet 1995). Fisher's exact tests were performed (GENEPOP 3.3d) to test for differences in allele frequencies between populations as an additional indicator of population subdivision, with significance levels determined using the Markov chain method (dememorisation number = 10000, batches = 100, iterations = 10000). Benjamini and Hochberg correction (Benjamini and Hochberg 1995) was used to control for multiple testing. A phylogenetic tree was constructed based on Nei's D measurement of genetic distances using Phylip (Felsenstein 2004). Population genetic structuring was further investigated using the Bayesian assignment package STRUCTURE v2.1 (Pritchard et al. 2000), placing individuals into 2–27 populations (K) (burn-in 50 k replicates, simulation 150 k MCMC replicates, no. of replicates of each k = 20). The K with the greatest delta change in log-likelihood was estimated using the method of Evanno et al. (2005). Admixture was assumed to account for intraspecific gene flow and prior information on sampling sites was not used. The program DISTRUCT (Rosenburg 2004) was used to graphically display the membership coefficient of an individual for a sub-population, which represents the fraction of its genome that has ancestry in the sub-population, (Rosenburg 2004). Finally, FLOCK (Duchesne and Turgeon 2009) was used to identify individuals of River Test origin stocked from the EA Calverton hatchery into the River Dove (750 individuals at two sites in 2007) River Wharfe (4000 individuals at two sites in 2008) and River

Table 2 Ten loci selected for screening with GenBank accession numbers, repeat motif, primer sequence and fragment size

Locus	Accession number	Repeat motif	Primer sequence	Fragment size	Multiplex	Dye	Original references
OGO2	AF009794	GA	F-TGTAAAACGACGGCCAGTACATCGCACACCATAAGCAT R-CGCATGTTTCCTCTGTGTTGAG	245–259	1	Fam	Olsen et al. (1998)
BFRO005	AF115407	CA	F-TAGAAGGCACAGTCGAGGCGCATCTGTATGAAAAACCT R-GGTTTGGTAGGAGTTTCGT	131–135	1	Ned	Susnik et al. (1999a)
BFRO018	AF175252	GT	F-TAGAAGGCACAGTCGAGGAGAGGGGTCCAGCAACATCA R-GGGGAACCAGTCTAAAGCCT	191–243	1	Ned	Susnik et al. (1999b)
BFRO011	AF130410	GT	F-GCAGGAAACAGCTATGACCATGGTTGATTGTGGGGGGA R-AACATCCTTACGCCCTAGCA	103–123	1	Pet	Susnik et al. (unpublished)
ONE2	U56700	GA	F-GCAGGAAACAGCTATGACGGTGCCAAGGTTCAGTTTATGTT R-CAGGAATTTACAGGACCCAGGTT	231–266	1	Pet	Scribner et al. (1996)
BFRO015	AF175249	CA	F-CCGCTCTAGAACTAGTGGACTCAGTGAAGAACTAAAGTACA R-GAAAAGTTATGAAGGTCAACCC	158–160	2	Vic	Susnik et al. (1999b)
BFRO010[a]	AF130409	CA	F-GCCGCTCTAGAACTAGTGTGGAGTGCAACGACCACGA R-GTGGTCTTGACTCAAGCATGAATC	290–304	2	Vic	Susnik et al. (unpublished)
BFRO016	AF175250	GT	F-TAGAAGGCACAGTCGAGGGCACACAATCTCTCTGTGAGTA R-ATCAGCCCAAGGTTGTAACA	181–187	2	Ned	Susnik et al. (1999b)
BFRO006	AF115408	GT	F-TGTAAAACGACGGCCAGTGCTTGGTTTTACCCTTTAGA R-AGGCATTTTACACTGGCATT	155–163	2	Fam	Susnik et al. (1999a)
BFRO012[a]	AF130411	CA	F-TGTAAAACGACGGCCAGTGTGGGATTTCTGCACATCCAAAGC R-TCCTGGCAGACTAGATACAATCCC	228–246	2	Fam	Susnik et al. (unpublished)

Two multiplex reactions were developed for high throughput screening. The multiplex and associated fluorescent dye are provided for each locus

[a] Indicates where primers were redesigned to prevent overlapping loci in the multiplex. Tail sequences are underlined. Original publication of each primer is provided

Aire (400 individuals at three sites from 2006 to 2008) with K = 2, 50 runs.

Control region analysis

The mitochondrial control region (D-Loop) was chosen as an informative marker based on previous work on European populations of grayling (Uiblein et al. 2001; Weiss et al. 2002; Gum et al. 2005). DNA extracts from 4 to 5 individuals from each of the 27 sampled UK populations were PCR-amplified using newly designed primers that amplify 825 bp of the mitochondrial D-loop corresponding to positions 41–866 of gbAF522422.1 (D-Loop Fwd: 5′-CCACCCTTAACTCCCAAAGCTAAGA-3′ and D-Loop Rev: 5′-TGGTTTAGGGGTTTGACAGGAA-3′). PCR reaction conditions and thermocycling parameters were: 20 µl reaction containing 0.33 units of ABgene Thermo-Start DNA Polymerase, 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 1× reaction buffer, 20 pmol each primer and 2–10 ng of template DNA. PCR was performed on a PTC-200 MJ Research thermocycler using the following cycling parameters: 15 min denaturation step at 95°C; 35 cycles of 94°C for 90 s, 55°C for 90 s, and 72°C for 90 s; 30 min elongation step at 72°C. Amplification products were visualised on an ethidium bromide-stained 2% agarose gel. Amplification products were then cleaned using exonuclease I (New England Biolabs) and shrimp-alkaline phosphotase (Invitrogen) (Werle et al. 1994). Bidirectional sequencing was carried out using BIGDYE version 3.1 chemistries on an ABI 3730xl genetic analyser (Applied Biosystems).

D-loop sequences were visualised and edited using Chromas 1.6 (Technelysium Pty Ltd). Prior to analyses sequence-similarity searches were performed using GenBank BLASTn tools (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences were aligned by eye using a European grayling voucher sequence labelled vou1 (AY594181). Evolutionary relationships were examined by constructing a haplotype network of the UK samples based on the statistical parsimony method of Templeton et al. (1992) performed in TCS (Clement et al. 2000). To determine the phylogenetic relationship between UK and European grayling the UK haplotype sequences were aligned with complete European haplotype sequences from Weiss et al. (2002). Phylogenetic inference was performed using Bayesian Inference (BI) implemented in MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). Bayesian analysis consisted of two independent runs each of 10 chains sampling every 1000 generations for 10 million generations using the GTR + I + G model. Convergence was assumed when the standard deviation of split frequencies was less than 0.01 and confirmed by the random plot of the generation versus the log probability of the data (the log likelihood values) as suggested in the program guidelines. The first 25% of the trees were disregarded as burn-in while the remaining trees were used to provide a 50% majority-rule consensus tree with associated branch lengths and posterior probabilities. The consensus tree was rooted using *T. arcticus* (AY168364.1).

Results

Microsatellites

Within population diversity

Multi-locus genotype information was obtained from all 1183 grayling distributed among the 27 river populations for 10 loci. Amplification success was high, ranging from 99.32% (BFRO011 and BFRO012) to 100% (BFRO005). Loci were moderately polymorphic in most of the populations, with the total number of alleles ranging from 2 (BFRO015) to 17 (BFRO018), while overall mean allelic richness (N_A) was 2.49 with population N_A ranging from 1.60 (SCA) to 3.47 (DEE) (Table 1).

Significant deviations from Hardy–Weinberg equilibrium were observed in 14 of 224 tests prior to correction for multiple tests, two of which remained significant after Benjamini and Hochberg correction (Benjamini and Hochberg 1995). Of 1215 pair-wise locus combinations across the 27 populations, two pairs (0.17%) exhibited linkage disequilibrium (LD). However, as no locus combinations were consistently in LD in all populations, these loci were retained for subsequent analyses. Inbreeding estimates (Fis) ranged from −0.12 (ERN) to 0.24 (ANN) (Table 1). The high Fis values observed in two 'introduced' populations (ANN, TWE) may reflect inbreeding resulting from founder effects (Mayr 1954). Mode-shifts were taken as evidence of founder effects/bottlenecks in ten populations: three 'native' (DEE, WLA, WLB), two 'native-stocked' (WRF, HAV) and five 'introduced' (CLD, EDN, GRF, TWE, KEN). Of these ten populations, four also showed a significant heterozygosity excess using a Wilcoxon sign rank test (WLA, $P = 0.027$; WLB, $P = 0.004$; HAV, $P = 0.00$; CLD, $P = 0.006$; KEN, $P = 0.004$).

Effective population sizes were small (Table 1) for all populations except for WYE and EDN, ranging from 12.0 in GRF (95% CI 10.0–17.8) to 121 in WYE (95% CI 81.6–372.7) A Kruskall-Wallis non-parametric test across all populations revealed that population classification (introduced, native, native-stocked) had a significant effect on number of alleles ($P = <0.014$), with post-hoc comparisons revealing a significant difference between native vs introduced (N_A native = 3.0 N_A introduced = 2.35, two tailed $P = 0.003$) Population classification did not have a significant effect on expected heterozygosity.

Population divergence

Pair-wise population differentiation measured by Theta was statistically significant (G-statistic, $P = <0.05$) for all comparisons except between IRF and WYE ($P = 0.63$) (Table 4). Fisher's exact tests revealed the same pattern of population differentiation, with significant pairwise differences in allele among all populations except IRF and WYE (data not shown). The un-rooted phylogram constructed from microsatellite frequencies using Nei's D (Fig. 2) suggests four main groups are present within the UK. Group A is composed of native Welsh populations (WYE, IRF and SEV) and ANN, suggesting ANN may have been introduced from a Welsh population. Group B included most Northern English populations (YKD, RIB, PKB, WRF, URE, AIR), Group C contained only Southern English populations (ITH, TST, HAV, WLA, WLB), while Group D contained all remaining populations in the UK including one Welsh (DEE), two Southern (DRF, KEN), two Midland (DOV, DBD), two Northern (DWB, EDN) and all Scottish populations except ANN (ERN, SCA, GRF, CLD, TWE).

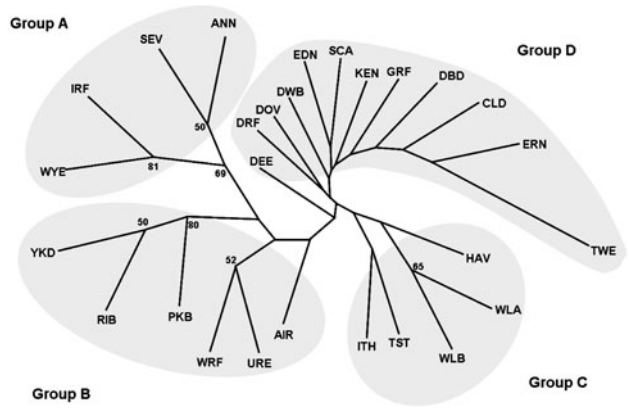

Fig. 2 Phylogram of population level relatedness based on Nei's D measurement of genetic distance. Bootstrap support (based on 2000 replications) is shown

The STRUCTURE analysis of microsatellite data indicated that the 27 populations grouped into five distinct clusters, with the ΔLnP(D) showing a clear peak at K = 5 (20.9) (Fig. 3a). The STRUCTURE analysis split Group D into two separate clusters, one (Group D1) contained most of the Scottish populations (ERN, GRF, SCA, CLD), one Northern (EDN), one Midland (DBD) and one Southern population (KEN) (Fig. 3e), and the second (Group D2) contained the remaining populations, one from each of the Welsh (DEE), Southern (DRF), Midland (DOV), Northern (DWB) and Scottish populations (TWE) (Fig. 3f). Groups A, B and C cluster according to geographic proximity, except in the case of the Annan (discussed below).

Flock analysis (Duchesne and Turgeon 2009) on three populations known to have been relatively recently stocked by the Calverton hatchery (AIR, WHF and DOV) with brood stock originally collected from the Test identified three stocked individuals in the DOV sample, one stocked

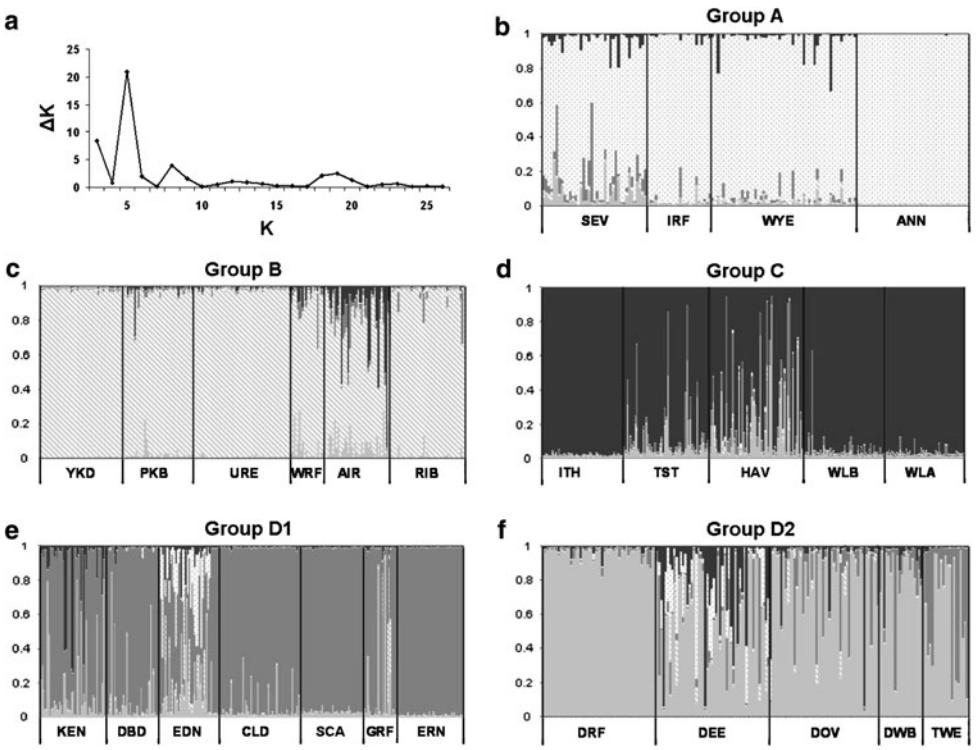

Fig. 3 STRUCTURE graphic showing grouping of populations based on 10 microsatellites. **a** Based on the method of Evanno et al. (2005) K was chosen with the greatest delta change in log-likelihood which was five. **b–f** Five groups identified by STRUCTURE. Each individual is represented by a single vertical line broken into five shaded segments, with lengths proportional to each of the five inferred clusters. Population codes are provided

individual in the AIR sample and no stocked individuals in the WRF sample.

D-Loop sequences

A total of seven variable sites and one indel were observed in 737 bp of the mtDNA D-Loop region from the 27 populations, allowing identification of 10 UK haplotypes (labelled H1-H10 Table 3). Haplotypes 1 and 5 have previously been reported in Europe (At18 and At21), corresponding to samples collected from the Rhine, the Ourthe and the Moselle (Weiss et al. 2002). Based on the five individuals per population that were sequenced, the dominant haplotype H1, occurred in all but two populations, H2 occurred only in the Welsh (IRF, WYE) and Scottish (ANN) and Northern (EDN) populations, H3 was observed in the Southern (ITH, TST, HAV, WLB) and one Welsh population (DEE), H4 and H7 were unique to SEV, H5 only occurred in AIR and RIB while all other haplotypes (H6 to H10) were unique to individual populations (Table 3). The DEE and WYE populations showed the greatest haplotype diversity, each exhibiting three haplotypes with H6 unique to DEE and H10 unique to WYE. Of the 27 populations, 15 exhibited no within-population diversity, with 13 containing only H1 while IRF and ANN contained only H2. Of these invariable populations, four were classified as native (YKD, PKB, IRF, WLA), eight were introduced (KEN, DRF, CLD, SCA, GRF, ERN, TWE, ANN) and three were native-stocked (WRF, DBD, DOV). A haplotype network of the UK samples identifies three main groups based within the data set (Fig. 4).

A Bayesian 50% majority rule consensus tree was constructed using the UK samples and additional samples from continental Europe (Fig. 5). Our analysis suggests that the UK samples fall within the group Weiss et al. (2002) labelled as 'Mixed Central Europe' and included haplotypes from the Rhine, Rhone and Denmark.

Discussion

Our results demonstrate that UK grayling display pronounced genetic structuring and restricted connectivity

Table 3 Variable nucleotide positions between the 10 UK grayling haplotypes and frequency in each population

	116	220	275	565	567	624	627	764	WRF	YKD	DWB	URE	AIR	PKB	KEN	TST	ITH
vou2	G	C	C	G	C	T	T	G	–	–	–	–	–	–	–	–	–
H1	.	.	.	.	.	.	.	A	1.00	1.00	0.80	0.60	0.80	1.00	1.00	0.80	0.25
H2	A	.	H	.	.	.	.	A	–	–	–	–	–	–	–	–	–
H3	.	.	.	.	.	–	C	A	–	–	–	–	–	–	–	0.20	0.75
H4	.	.	T	A	T	.	.	A	–	–	–	–	–	–	–	–	–
H5	.	.	T	.	.	–	C	.	–	–	–	–	0.20	–	–	–	–
H6	.	.	T	.	.	.	.	A	–	–	–	–	–	–	–	–	–
H7	.	.	T	.	T	.	.	A	–	–	–	–	–	–	–	–	–
H8	.	T	T	.	.	.	C	.	–	–	–	0.40	–	–	–	–	–
H9	.	.	.	.	.	.	.	A	–	–	0.20	–	–	–	–	–	–
H10	A	.	T	.	T	.	.	A	–	–	–	–	–	–	–	–	–

	DRF	HAV	WLA	WLB	WYE	DEE	IRF	DBD	DOV	SEV	EDN	RIB	ERN	ANN	CLD	GRF	TWE	SCA	Accession number
vou2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	AY594182
H1	1.00	0.50	1.00	0.80	0.20	0.20	–	1.00	1.00	–	0.75	0.80	1.00	–	1.00	1.00	1.00	1.00	GQ982905
H2	–	–	–	–	0.60	–	1.00	–	–	–	0.25	–	–	1.00	–	–	–	–	GQ982906
H3	–	0.50	–	0.20	–	0.40	–	–	–	–	–	–	–	–	–	–	–	–	GQ982907
H4	–	–	–	–	—	–	–	–	–	0.60	–	–	–	–	–	–	–	–	GQ982908
H5	–	–	–	–	–	–	–	–	–	–	–	0.20	–	–	–	–	–	–	GQ982909
H6	–	–	–	–	–	0.40	–	–	–	–	–	–	–	–	–	–	–	–	GQ982910
H7	—	–	–	–	–	–	–	–	–	0.40	–	–	–	–	–	–	–	–	GQ982911
H8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	GQ982912
H9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	GQ982913
H10	–	–	–	–	0.20	–	–	–	–	–	–	–	–	–	–	–	–	–	GQ982914

The nucleotide numbering system follows that of vou2

between all but a few populations. Despite the high degree of differentiation among the 27 populations sampled, analyses of microsatellite data reveal four or five groups (depending on the analysis method) that, with the exception of two populations, group largely by geographic location. Breakdown of the relationship between geographic sampling location and genetic group is likely to be a result of anthropogenic movement during the last 200 years. However, while contemporary natural gene flow between populations appears restricted, lack of monophyly at the mtDNA D-Loop suggests historical connectivity, possibly reflecting post-glacial colonization from Europe. Our results also suggest that many populations have undergone genetic bottlenecking, which may be due to a lack of genetic variability in founding populations, subsequent population declines or genetic drift. Effective population sizes were found to be small in all populations with only seven populations exceeding an Ne of 50 by any margin. Based on highly significant differences in allele frequencies at microsatellite loci, we identify potential Management Units (MUs) in the UK populations of grayling and provide recommendations for conserving natural genetic diversity.

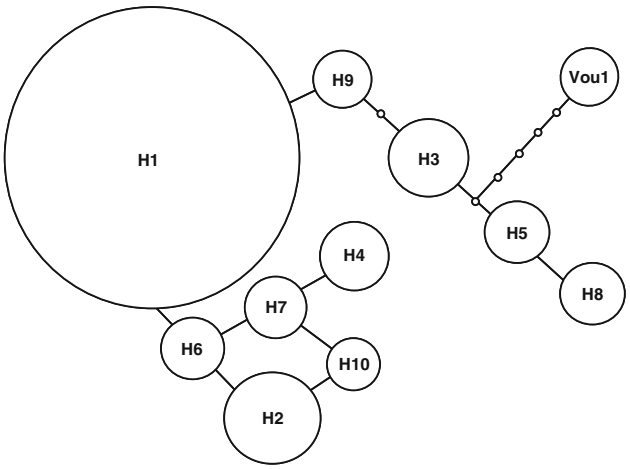

Fig. 4 Haplotype network of UK samples including grayling voucher sequence (vou1)

Population structure

Genetic divergence among UK populations was high and this result is concordant with other studies on grayling in Europe (Gross et al. 2001; Koskinen et al. 2002a; Gum et al. 2005). All populations were significantly genetically differentiated based on microsatellite markers with the exception of the Wye and its tributary the Irfon. Clustering methods group the data into either 4 or 5 groups of populations depending on the clustering method. The

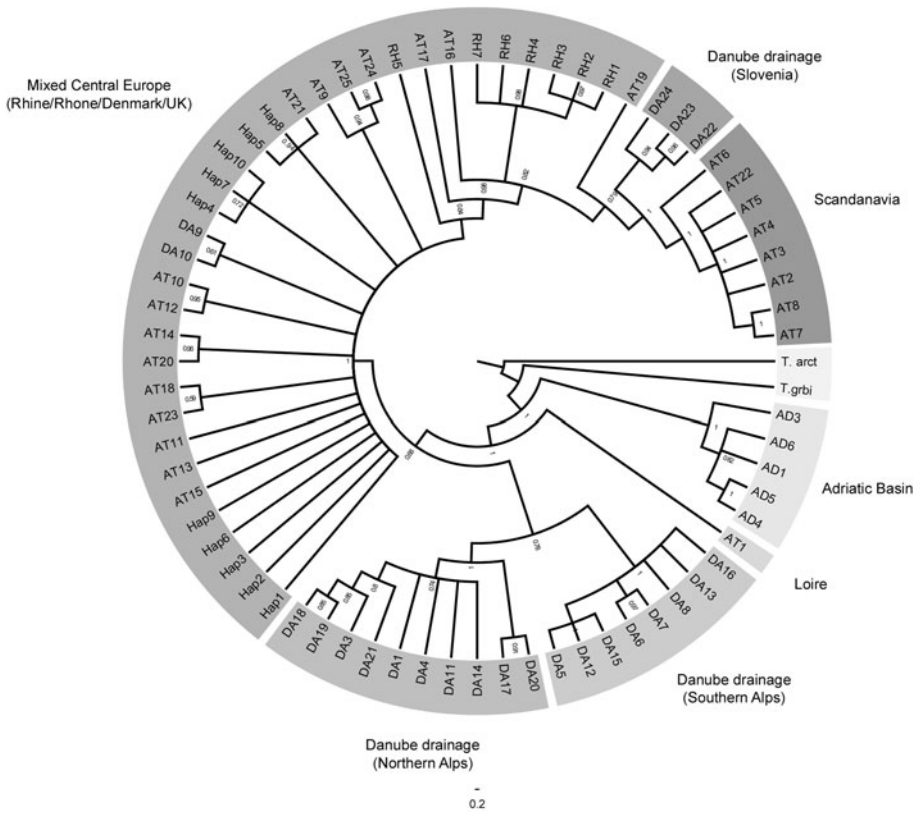

Fig. 5 Mitochondrial D-loop phylogeny of UK and continental European samples. UK haplotypes labelled Hap1-Hap10. European samples and clade nomenclature taken from Weiss et al. 2002. Node support given as posterior probabilities

neighbour joining phylogram identified four main clusters, whereas STRUCTURE identified five clusters by splitting group D in two. Close inspection of the neighbour joining tree also reveals a split between these two subgroups, but with low support. Consequently we have labelled the subgroups D1 and D2 rather than assigning them to separate clusters.

Genetic evidence of introductions and stocking

The current distribution of freshwater fish species in the UK has been greatly influenced by the stocking and transfer of individuals by man (Wilson 1966; Wheeler 1977; Ibbotson et al. 2001). Stocking of grayling in the UK has occurred sporadically over the last 200 years and can be categorised as either historical (usually anecdotal evidence of stocking pre 1900) or more recent (from EA fish rearing units or stock translocations where alleged "excess" grayling have been moved to recipient rivers). Currently, populations are supplemented with fish sourced from the EA fish farm at Calverton where 12–20 thousand fish per year are reared from brood stock originating from three rivers in the Midlands region: the Derwent, Dove and Derbyshire Wye (Ibbotson et al. 2001), and the Test in the South. Angling literature states that "few rivers escaped the grayling stocking fever that swept the UK during the seventies and eighties of the 19th Century" (Wilson 1966). Historically, rivers were stocked to provide sport for anglers and not for conservation purposes. More recently, emphasis has shifted toward the sustainability of populations through effective management and conservation.

To a large extent, records of population introductions and stocking events support the patterns revealed by our molecular data. The Derbyshire Derwent and the Dove have been used as source stocks for many of the introductions in the last 200 years, for instance, angling literature states that the Clyde was originally stocked from the Derbyshire Derwent (Wilson 1963; Ibbotson et al. 2001). Accordingly, both the nuclear allele-frequency data and the mtDNA haplotype data group the introduced populations Clyde, South Calder, Gryffe, Earn, and Kennet with the Derbyshire Derwent and Driffield West Beck and Dorset Frome and Tweed with the Dove. The Itchen and Test group with their geographical neighbours in southern Group C. The Hampshire Avon was used to stock the Test and many other rivers in the area (Wilson 1963; Ibbotson et al. 2001), and it is therefore conceivable that it was also used to stock the Itchen. Most of the native-stocked populations group with their geographical neighbours, including the Wharfe, Aire and Ribble in Group B. The Hampshire Avon groups with the native Wylye populations in Group C and while there are no records of translocation between these populations, the Wylye is a tributary of the Hampshire Avon and the genetic similarity could have arisen through natural gene flow. Source individuals for more recent introductions from the Calverton Hatchery have originated from the River Test. Each year, wild individuals are caught from the Test, stripped and the offspring raised within the hatchery. Stocking records indicate that the Aire, Wharfe and Dove have received large numbers of individuals from the Test since 2006. We identified one stocked individual within our Aire sample and three within the Dove sample that were likely to be of Test origin, suggesting that some stocked individuals are surviving within these rivers. However, no individuals within the Wharfe sample were assignable to Test origin. Genetic evidence contradicted anecdotal information that the Annan was stocked from the Derbyshire Derwent (Wilson 1963). Based on nuclear allele frequencies and mtDNA, however, the Annan consistently grouped with the Welsh populations in Group A suggesting that the Wye or Irfon, rather than the Derbyshire Derwent, was likely the source population for the Annan.

Population bottlenecks and effective population size

A consequence of sudden dramatic population decline is a reduction in average heterozygosity as genetic variability is lost (Nei et al. 1975). A similar effect can also be observed in introduced populations when founding individuals are not genetically diverse or in sufficient number (Mayr 1954). Genetic bottlenecks were identified in ten populations, three classified as 'native' (Dee, Wylye-A, Wylye-B), two as 'native-stocked' (Wharfe, Hampshire Avon), and five as 'introduced' (Clyde, Eden, Gryffe, Kennet, Tweed). In the native Dee anecdotal evidence suggests that this population has been declining since the late 20th Century, possibly due to predation by piscivorous birds (Ibbotson et al. 2001). In certain areas of the river Wylye grayling were historically removed in an effort to control the population (Ibbotson et al. 2001) which may have contributed to the observed decline in genetic diversity. Among the native-stocked populations, bottlenecking detected in the Wharfe may be explained by the reported decline in numbers since the 1980s (Ibbotson et al. 2001), while the Hampshire Avon was thought to have sustained an abundant grayling fishery (Ibbotson et al. 2001). The reduced ability of bottlenecked populations to respond to environmental perturbations (Frankham et al. 2002) underlines the importance of considering genetic processes when managing fisheries. The five introduced populations may simply show bottlenecking as

a consequence of the transfer of relatively few founding individuals or the transfer of many individuals from a population that had previously undergone a bottleneck event. Without historical samples for pre- and post-stocking comparison, however, the cause of the observed bottleneck remains conjectural.

Effective population sizes (estimated using ONESAMP) were small and less than 50 for 19 of the 27 populations investigated, and only one had a mean Ne greater than 100 (Wye = 121). These results are similar to those found by Swatdipong et al. (2010) who also found most of the populations they investigated to have effective population sizes less than 50. These small sizes have important conservation implications as populations with an Ne of less than 50 are widely considered to be at risk of inbreeding depression (Franklin 1980; Rieman and Allendorf 2001). Due to the high site fidelity found among grayling, however, low Ne estimates may reflect the Ne of a subset of the population rather than that of the population as a whole (Swatdipong et al. 2010). To investigate this possibility in our UK data set, we combined the two population samples from the Wylye and estimated Ne using ONeSamp. Ne was 37.1 (95% CI 26.5–95.4) which is almost identical to the two separate Wylye estimates (33.5 and 39.7), suggesting that Ne estimates may be truly representing population rather than sample Ne. As a cautionary note, estimates of effective population size used here are based on a single point sample per population.

Origins of UK grayling

The UK samples analysed in the current study fall with strong support into the clade containing samples from the Atlantic basin labelled as 'mixed central Europe' in the study by Weiss et al. (2002) and 'mt lineage IIa' in the study by Gum et al. (2005). This basin includes the Rhine, Outhe and Moselle, where two UK haplotypes also occur (Weiss et al. 2002), suggesting that grayling in the British Isles originated from these rivers and probably colonised the UK via the North Sea River, a theory put forward for many British fish species (Regan 1911; Wilson 1963; Wheeler 1977). Our data support the idea that southern England may indeed have been the route of UK colonisation, given that populations in this area contain haplotypes that are basal and seen elsewhere in Europe. However, our data do not rule out the possibility that northern England may have served as a second route of colonisation, since populations possess both the basal and an additional European haplotype, The lack of pristine native UK populations, free of stocking, weakens inferences about specific routes of colonisation into the UK.

Recommendations for management of UK grayling

Phylogenetic clarification of species assemblages has important implications in the conservation and management of threatened populations (Moritz 1994). Without a clear understanding of what constitutes a species, subspecies, population or subpopulation it becomes difficult to efficiently assign management resources and plan effective conservation strategies. Based on mtDNA data, UK grayling fall within the same clade as continental samples from the Atlantic drainage and therefore do not constitute a historically isolated unit that could be considered as a single ESU. Nevertheless, species are often managed at a national level notwithstanding ESU status. The only UK population that may constitute an ESU is the Severn, being the only population to consist entirely of unique haplotypes and therefore showing divergence at both nuclear and mtDNA genes. However, as only five individuals were sequenced, this inference is tentative. All other UK populations analysed in this study can be classified as separate Management Units (with the exception of the Wye and its tributary the Irfon), as they show statistically significant differences in allele frequencies from each other at nuclear microsatellite loci (Moritz 1994).

Previous studies on the conservation of grayling have suggested the existence of several ESUs within Europe (Gum et al. 2003; Gum et al. 2005), but many grayling populations also exist as separate MUs within ESUs (Koskinen et al. 2002a). Such studies have recommended that populations should be supplemented from distinct brood stocks, even across relatively short geographical distances (Koskinen et al. 2002a) or within drainages (Gum et al. 2009). The significant level of population structuring reported here (Table 4) is consistent with previous studies of continental grayling populations (Koskinen et al. 2002a; Gum et al. 2005) and, following the same recommendations as suggested for continental Europe, populations therefore should be stocked only from their own river (in line with EA augmentation policy 21 of the National Trout and Grayling Fisheries Strategy) or failing that, their nearest genetic relatives. This would ensure that the genetic distinctness of the Welsh populations (Group A), the northern populations (Group B), the southern populations (Group C) and the remaining UK populations (Groups Da and b) persists. However, we consider stocking to be an intervention of last resort and would prefer to see efforts targeting habitat improvement and exploitation control. We echo the recommendations of Youngson et al. (2003) that populations should be combined into fisheries-based management units comprised of interchangeable groups of populations. Such measures should conserve native genetic diversity while providing a practical approach to management.

Table 4 Population pair-wise Fst values and significance levels

	WRF	YKD	DWB	URE	AIR	PKB	KEN	TST	ITH	DRF	HAV	WLA	WLB	WYE	DEE	IRF	DBD	DOV	SEV	EDN	RIB	ERN	ANN	CLD	GRF	TWE	SCA
WRF	–	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
YKD	0.36	–	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
DWB	0.31	0.53	–	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
URE	0.14	0.42	0.46	–	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AIR	0.09	0.44	0.26	0.24	–	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
PKB	0.27	0.10	0.36	0.34	0.31	–	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
KEN	0.42	0.56	0.19	0.52	0.37	0.46	–	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
TST	0.26	0.46	0.12	0.38	0.19	0.33	0.19	–	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
ITH	0.33	0.52	0.33	0.42	0.27	0.39	0.44	0.12	–	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
DRF	0.34	0.53	0.26	0.43	0.29	0.40	0.36	0.19	0.31	–	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
HAV	0.24	0.41	0.16	0.35	0.22	0.30	0.18	0.12	0.29	0.27	–	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
WLA	0.36	0.52	0.31	0.46	0.31	0.42	0.35	0.23	0.32	0.39	0.11	–	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
WLB	0.30	0.49	0.20	0.42	0.25	0.38	0.25	0.13	0.24	0.32	0.06	0.04	–	*	*	*	*	*	*	*	*	*	*	*	*	*	*
WYE	0.34	0.46	0.41	0.44	0.39	0.40	0.47	0.40	0.47	0.42	0.33	0.38	0.37	–	*	NS	*	*	*	*	*	*	*	*	*	*	*
DEE	0.20	0.36	0.13	0.32	0.20	0.27	0.18	0.15	0.29	0.25	0.07	0.17	0.11	0.27	–	*	*	*	*	*	*	*	*	*	*	*	*
IRF	0.38	0.49	0.46	0.47	0.42	0.43	0.52	0.43	0.50	0.46	0.37	0.42	0.40	0.00	0.28	–	*	*	*	*	*	*	*	*	*	*	*
DBD	0.29	0.46	0.15	0.42	0.25	0.36	0.16	0.16	0.35	0.26	0.15	0.26	0.18	0.39	0.12	0.42	–	*	*	*	*	*	*	*	*	*	*
DOV	0.26	0.44	0.10	0.38	0.22	0.27	0.30	0.12	0.28	0.19	0.16	0.32	0.24	0.41	0.18	0.45	0.20	–	*	*	*	*	*	*	*	*	*
SEV	0.35	0.45	0.39	0.46	0.39	0.39	0.46	0.39	0.49	0.45	0.35	0.42	0.37	0.33	0.20	0.34	0.35	0.42	–	*	*	*	*	*	*	*	*
EDN	0.25	0.45	0.20	0.38	0.26	0.37	0.20	0.21	0.38	0.30	0.16	0.28	0.20	0.35	0.12	0.39	0.11	0.24	0.32	–	*	*	*	*	*	*	*
RIB	0.22	0.16	0.40	0.30	0.33	0.10	0.49	0.37	0.43	0.42	0.33	0.45	0.41	0.38	0.29	0.41	0.39	0.33	0.37	0.37	–	*	*	*	*	*	*
ERN	0.48	0.57	0.34	0.57	0.43	0.49	0.25	0.32	0.51	0.47	0.27	0.40	0.34	0.51	0.26	0.56	0.18	0.38	0.48	0.35	0.53	–	*	*	*	*	*
ANN	0.53	0.64	0.72	0.55	0.57	0.60	0.73	0.63	0.67	0.67	0.58	0.63	0.61	0.44	0.49	0.52	0.64	0.66	0.41	0.58	0.55	0.75	–	*	*	*	*
CLD	0.36	0.48	0.22	0.46	0.30	0.39	0.22	0.21	0.37	0.28	0.22	0.31	0.25	0.43	0.19	0.46	0.03	0.24	0.40	0.18	0.43	0.17	0.65	–	*	*	*
GRF	0.30	0.47	0.22	0.41	0.28	0.35	0.19	0.19	0.33	0.27	0.18	0.29	0.23	0.38	0.17	0.41	0.09	0.23	0.41	0.15	0.39	0.27	0.68	0.08	–	*	*
TWE	0.36	0.50	0.15	0.47	0.30	0.35	0.27	0.20	0.37	0.30	0.23	0.33	0.26	0.42	0.20	0.45	0.16	0.20	0.39	0.30	0.41	0.25	0.70	0.16	0.20	–	*
SCA	0.53	0.70	0.27	0.60	0.45	0.61	0.36	0.31	0.50	0.39	0.36	0.48	0.38	0.55	0.31	0.63	0.23	0.38	0.54	0.27	0.62	0.49	0.81	0.29	0.36	0.41	–

* Represents significance at the 0.05 level. NS = not significant

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