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Identification of reproductively isolated lineages of Amur grayling (*Thymallus grubii* Dybowski 1869): concordance between phenotypic and genetic variation

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Abstract

We analysed variation at maternally (mitochondrial DNA control region sequences) and biparentally (10 microsatellites) inherited genetic markers, as well as across 12 meristic characters in 7 populations of Amur grayling, *Thymallus grubii*, from eastern Siberia. All three data sets were concordant in supporting the existence of three diagnosable, reciprocally monophyletic, and most probably reproductively isolated, lineages of grayling within the Amur drainage. There was a significant correlation between genetic and phenotypic divergence, both within and among lineages. Two phenotypically distinct forms (with and without an orange spot on the posterior portion of the dorsal fin), found in sympatry in the lower Amur, most likely result from secondary contact, as they demonstrate 4.6% sequence divergence at the mitochondrial DNA control region. This divergence, together with the existence of at least one nearby population of orange spot grayling outside the Amur drainage (0.8% divergence) underscore the palaeo-hydrological complexity of the system, which presumably promoted genetic divergence in a shifting allopatric framework throughout the Pleistocene. Grayling from the upper Amur, corresponding to the type locality for the species, formed a sister group (1.4–1.6% divergent) to the orange spot lineage perhaps diverging in the early Pleistocene (1.4–1.6 Ma).

Keywords: Amur River, control region, grayling, microsatellites, *Thymallus grubii*

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Introduction

Freshwater organisms live in isolated or fragmented habitats that restrict gene flow among demes. Fishes of the family Salmonidae exhibit life history traits such as natal homing that further promote population structure, especially within the complex hydrological networks of the temperate ecosystems in which they are found. Such structure, in combination with the probable buffering effects and other characteristics of an autotetraploid ancestry (Allendorf & Thorgaard 1984), appears to promote intraspecific phenotypic variation. This diversity, combined with the popularity and economic value of many salmonids in commercial and sport fisheries, has attracted the attention of population geneticists and evolutionary biologists for decades. However, concordance between observed phenotypic and genetic divergence has often been illusory. Thus, one of the first broad-scale phylogeographical studies on the polytypic brown trout *Salmo trutta*, revealed five major matrilines, defined by primary catchment basins but wholly discordant with existing taxonomic epithets (Bernatchez *et al*. 1992; Osinov & Bernatchez 1996), which had historically led to the description of up to 50 species (Behnke 1986). In addition, studies evaluating sympatric forms of whitefishes *Coregonus* spp. (Shields *et al*. 1990; Turgeon *et al*. 1999; Lu *et al*. 2001; Rogers *et al*. 2001) often fail to reveal reproductive isolation and uncover complex patterns of parallel evolution that underscore both the short- (within generation) and long-term (across generation) plasticity of many salmonid lineages. Phenotypic and genetic diversity find more concordance within allopatric frameworks such as for cutthroat trout *Oncorhynchus clarkii* in western North America where no fewer than 14 taxa are recognized, each existing in disjunct intermountain basins (Behnke 1992). But for other subspecific designations within the genus, such as for *O.*

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mykiss in British Columbia, genetic data have provided little or no support (McCusker *et al*. 2000).

It is particularly illuminating that, despite the incongruence of major evolutionary (mitochondria; DNA) lineages of *S. trutta* with historical taxonomy, there have been numerous demonstrations of phenotypic or life history variants within a single lineage that demonstrate genetic differentiation and little or no gene flow. Among these, the most often cited involves three sympatric morphs within a single lake (Ferguson & Mason 1981; Ferguson 1986). In rivers, divergent life history strategies (resident vs. anadromous) are reported within populations of one major mitochondrial DNA (mtDNA) lineage (Hansen *et al*. 2000; Antunes *et al*. 2001). This may be typical for a geographically fragmented species, wherein mutations of adaptive significance, or at least those influencing phenotypically recognizable traits occur in a mosaic pattern with respect to major mtDNA lineages (see Discussion in Antunes *et al*. 2001). For lake-dwelling coregonids (Bernatchez *et al*. 1999) and charrs (*Salvelinus*) (Jonsson & Jonsson 2001) recurrent ecologically driven evolution frequently results in sympatric ecomorphs, similar to patterns seen in other freshwater fishes such as three-spine stickleback *Gasterosteus aculeatus* (McKinnon & Rundle 2002). While experimental work (Skúlason *et al*. 1993), sometimes combined with the application of highly variable genetic markers (Bernatchez *et al*. 1999), has demonstrated a genetic basis for at least some differences in sympatric morphs, from a taxonomic perspective, it must still be emphasized that the weak concordance between phenotype and genotype across populations confounds or prevents application of classical systematics (Jonsson & Jonsson 2001).

Compared with the salmonid genera listed above, *Thymallus (*grayling) has not been noted for extraordinary phenotypic diversity. This is strikingly apparent for Europe, where only a single noncontroversial taxon exists for the widespread European grayling, *T. thymallus* (Kottelat 1997), whereas in this same systematic review the numbers of potential species for *Coregonus* and *Salmo*, under application of a Phylogenetic Species Concept (PSC), have been increased to at least 44 and 27, respectively. Notwithstanding the resistance to adopting such a PSC framework for salmonid systematics, one must ask if grayling are really so monomorphic compared with other salmonids, or is their simplicity simply a reflection of a lack of attention? This question is particularly relevant in the light of recent molecular-based studies which demonstrate that demes of *T. thymallus* within a single lake (Lake Saimaa Finland; Koskinen *et al*. 2001, 2002a) or the upper Danube (Weiss *et al*. 2002) display not only stark structure, but also considerably deeper genetic divergence than co-occurring *S. trutta*. Clear distinctions between upper Danubian and Main and Elbe populations of *T. thymallus* are also concordant with these above-cited studies (Gross *et al*. 2001). Although the reasons for the high divergence estimates in *T. thymallus* are not entirely clear, it has been suggested that extreme site fidelity or low dispersal capacity may be important factors (Koskinen *et al*. 2001, 2002a).

Similar to populations of *Salmo*, *Salvelinus* and *Coregonus* in Europe, *Thymallus* populations have suffered from anthropogenic change and manipulation (Persat 1996; Uiblein *et al*. 2001), and thus future attempts to evaluate phenotypic and genetic concordance, or systematic revision may be difficult. This is especially true for southern populations in which introgression from translocations are documented (Persat 1996; Uiblein *et al*. 2000, 2001; Weiss *et al*. 2002) but both phenotypic (Jankovic 1962) and genetic uniqueness have also been noted (Sušnik *et al*. 2001), with the latter study arguing for distinct taxonomic status of Adriatic populations. It is in this general framework that the investigation of *Thymallus* in a large undisturbed system becomes attractive.

Such a system exists in Siberia, Russia. The Amur river is an enormous hydrological system with a complex palaeohistory (Grosswald 1998) and grayling occupy tributary systems from its headwaters in Mongolia and eastern Siberia, to its mouth through the Tatar Strait opposite Sakhalin Island, as well as nearby isolated tributary systems of the Okhostk Sea and Sea of Japan. Although the Amur itself is far from pristine, salmonid fish culture and translocations are unheard of, there are no dams on its main channel, and most of the cold-water tributary habitats occupied by grayling are relatively pristine and distant from the more populated settlements along the river.

Amur River grayling were first described at the specific level *Thymallus grubii* (Dybowski 1869) based on collections in the upper basin, a designation accepted by Pivnicka & Hensel 1978). Nonetheless, this status has been disregarded in at least some western (Scott & Crossman 1998) as well as Asian literature (Berg 1949) and thus grayling of the Amur basin are variously referred to as *T. grubii* or a subspecies of Arctic grayling *T. arcticus grubii* (historically misspelt as *grubei*). Moreover, DNA–DNA hybridization data (Skuirikhina 1984) and morphological investigations (Tugarina & Khramtzova 1980; Shatunowsky 1983) have indicated significant differences between some grayling from the upper and lower Amur. Recently, Koskinen *et al*. (2002b) reported mean divergence estimates (4.3–5.5%) at the mitochondrial control region and portions of flanking transfer RNA (tRNA) regions for two populations of *T. grubii* in the upper Amur basin compared with Arctic grayling (*T. arcticus*) from the upper Lena basin, Lake Baikal and its tributaries, the upper Enisey basin, as well as several individuals from North America. Furthermore, using European grayling *T. thymallus* as an arbitrary outgroup in that study, *T. grubii* was the sister taxa to the lineages of *T. arcticus* found in the above-mentioned drainages. Significant genetic differentiation between Asian (Yana

basin) and North American populations of *T. arcticus* has also been shown in Redenbach & Taylor (1999).

In this study, we used a comprehensive approach to investigate the evolutionary diversity of Amur basin grayling. General phenotype (coloration) and meristic data were combined with both multilocus nuclear data (microsatellites) and mtDNA sequence data to ascertain the diagnosability of several putative forms of grayling occurring in the Amur, including one neighbouring Pacific drainage population. Combined use of mtDNA sequence and microsatellite variation was also used to place these potential taxa into an evolutionary framework, and to evaluate the level of reproductive isolation.

Materials and methods

Grayling (N = 207) were collected by angling in 1998–2001 from seven locations in eastern Siberia, primarily within the Amur drainage (see Table 1, Fig. 1). Based on a comment by Antonov *et al*. (1996), fish were first noted as to

Table 1 Sample locations including major river basin, geographical coordinates and the number of individuals analysed for mtDNA, microsatellite (Msats) and meristic variation

			No. individuals					
			mtDNA					
Population	Basin	PopCode	Sequences	RFLPs	Msats	Meristics	Latitude (N)	Longitude (E)
Onon River*	Shilka→ Amur	Amu (1)	5	—	41	22	48°35′	110°48′
Sypchegurka River*	Ingoda→ Amur	Amu (2)	4	—	—	34	51°20′	113°26′
Olengui River	Ingoda→ Amur	Amu (2)	—	—	—	28	51°02′	112°34′
Uber Zhelykhen River	Ingoda→ Amur	Amu (2)	—	—	—	32	50°48′	112°17′
Annui River†	Annui→ Amur	Anu (3–5)	23	70	57	74	49°17′	137°55′
Merek River	Amgun′→Amur	Mer (6)	7	1	7	8	51°17′	134°47′
Buta River	Khutu→ Tumnin	But (7)	6	3	6	8	49°09′	138°56′

*Sequences from these samples derive from Koskinen *et al*. 2002b, where they were coded 'Amu' for Amur basin; this nomenclature is retained here to avoid confusion with existing GenBank entries.
†These samples include fish caught in the Gobili and Ertukuli rivers, at their confluence with the Annui River.

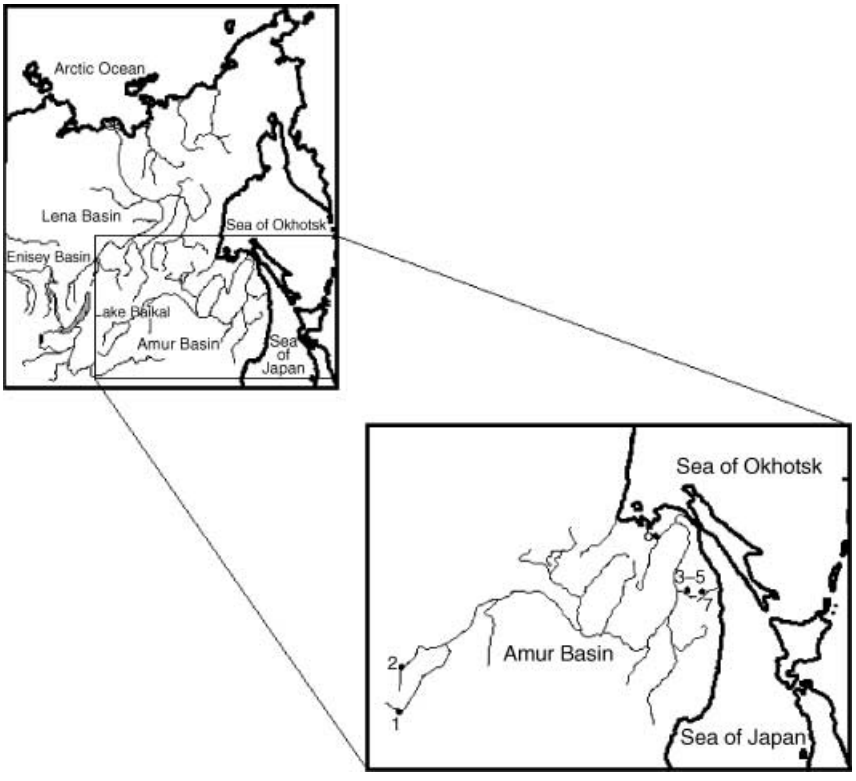

Fig. 1 Map of the Amur River (in detail) indicating the geographical sample locations of the *Thymallus grubii* populations analysed in this study. For population names and codes see Table 1.

Table 2 Number of individuals, means and SD in parentheses for standard length (mm) and the 12 meristic characters used to characterize the three major phenotypes; upper Amur, lower Amur unspotted, and lower Amur orange spotted. Results of the Kruskal–Wallis test for meristic characters are shown as the maximum mean difference in ranks, the chi-square value, and the *P*-value

	Phenotype/Population			Kruskal–Wallis test	
Character	Upper Amur (N = 117)	Lower Amur (N = 57)	Lower Amur Orange (N = 33)	max. Δ in mean rank (Chi square)	P-value
SL	168.0 (20.3)	196.1 (26.6)	221.5 (36.8)		
(i) Lateral line pores	90.8 (3.9)	81.6 (3.0)	91.0 (4.7)	96.8 (106.4)	< 0.001
(ii) Unbranched dorsal rays	8.2 (0.8)	9.4 (0.9)	10.1 (0.8)	95.5 (97.7)	< 0.001
(iii) Branched dorsal rays	12.6 (0.9)	15.5 (1.0)	13.2 (0.8)	103.5 (121.5)	< 0.001
(iv) Total dorsal rays	20.8 (0.9)	24.9 (0.7)	23.3 (0.9)	114.2 (154.6)	< 0.001
(v) Branched pectoral rays	13.9 (0.5)	14.2 (0.7)	14.4 (0.7)	36.6 (14.7)	= 0.001
(vi) Ventral rays	9.1 (0.4)	10.0 (0.3)	9.2 (0.4)	84.3 (103.9)	< 0.001
(vii) Unbranched anal rays	4.1 (0.4)	4.2 (0.4)	4.3 (0.5)	23.6 (6.9)	= 0.031
(viii) Branched anal rays	9.0 (0.6)	9.0 (0.4)	9.1 (0.5)	11.6 (2.0)	= 0.356
(ix) Gill rakers	17.8 (1.3)	18.2 (1.1)	18.9 (1.0)	48.1 (17.6)	< 0.001
(x) Branchiostegal rays	9.7 (0.6)	9.9 (0.6)	9.6 (0.6)	27.9 (7.3)	= 0.026
(xi) Vertebrae	55.3 (0.9)	53.3 (0.8)	54.9 (1.0)	91.7 (96.0)	< 0.001
(xii) Pyloric caeca	15.0 (2.0)	14.6 (1.4)	17.1 (2.6)	59.0 (22.9)	< 0.001

whether they had an obvious yellow–orange ocellus-like spot on the posterior edge of the dorsal fin. After a small fin clip had been preserved in 96% ethanol, whole fish were fixed in a 4% formalin solution.

Meristic analysis

Phenotypic characterization was based on 12 meristic characters: (i) pores along the lateral line, (ii) unbranched dorsal rays, (iii) branched dorsal rays, (iv) total number of rays, (v) branched pectoral rays, (vi) ventral rays, (vii) unbranched anal rays, (viii) branched anal rays, (ix) gill rakers on the first left branchial arch, (x) branchiostegal rays, (xi) vertebrae (with central and haemal spines), and (xii) pyloric ceacea (Table 2). All counts were made on fixed material following methodology in Pravdin (1966) with the following modifications: vertebrae count does not include the urostyle; gill raker and lateral line pore counts are all-inclusive aided by a 8–16× binocular; and the last branched ray in the dorsal and anal fins, which looks like two separate rays, is counted as one. A Spearman correlation coefficient was used to test which, if any, meristic characters were significantly correlated with size. A principal component analysis (PCA) was carried out on the correlation matrix (i.e. standardized data) of raw data, as at least one variable (number of pores) had a considerably higher variance than the others (Johnson 1998). Extracted factors were plotted against one another in order to evaluate the value of the analysis in delineating populations or phenotypes. A canonical discriminant analysis (CDA) was used to quantify the discriminatory power of these factors. Prior probabilities of inclusion were considered equal for all groups. Extracted factors were also used to create a distance matrix (Euclidean distances) between individuals, in order to evaluate correlation between genetic and morphological distances (see below). PCA and other univariate statistics were performed using the R-package (Casgrain & Legendre 2001) and the CDA was carried out in SPSS 9.0.

mtDNA analysis

The complete mtDNA control region and a partial segment of both flanking tRNA genes were amplified in 119 individuals using the LRBT-25 and LRBT-1195 primers first described in Uiblein *et al*. (2001) and used in Weiss *et al*. (2002). The mtDNA data for two upper Amur tributaries (N = 9) (Onon and Sypchergurka) and the two outgroup samples from Europe are from Koskinen *et al*. (2002b). Polymerase chain reactions (PCR) were carried out in 25 μL volumes. Each reaction contained 19 μL H_2O, 2.5 μL 10× Promega buffer B, 0.5 μL 10 mM of each primer, 1.5 μL 25 mM $MgCl_2$, 0.5 μL 10 mM dNTPs, 0.1 μL Promega *Taq* DNA polymerase, and 0.5 μL 100 ng/μL DNA template. The PCR conditions were as follows: initial denaturation at 94 °C for 3 min, followed by denaturation at 94 °C (45 s), annealing at 55 °C (45 s) and extension at 72 °C (45 s) repeated for 30 cycles. Amplified DNA templates were purified using the QIAquick PCR Purification Kit (Qiagen) and ≈ 100 ng of purified PCR product from 45 individuals (see Table 1) were used in cycle sequencing reactions following ABI PRISM BigDye Terminator protocols. Sequences were visualized on an ABI-310 and aligned by hand with the aid of comparative alignments in SEQUENCE NAVIGATOR software. New mtDNA sequences generated in this study have been deposited in GenBank under Accession nos AY246389–AY246424. The remaining 74

individuals were assessed using random fragment length polymorphism (RFLP) methodology developed after the initial sequences were analysed (see Results).

Both maximum parsimony (MP) and maximum likelihood (ML) were used for phylogenetic reconstruction in the program PAUP Version 4.0b10 (Swofford 2001). For MP, insertions or deletions (indels) were included as a fifth character. A heuristic search (10 replicates) with TBR branch-swapping was employed to find the most parsimonious trees. For ML, a sequence evolution model was first chosen using the program MODELTEST Version 3.06 (Posada & Crandall 1998) incorporated into PAUP. After a model was chosen, a heuristic search (10 replicates) was used to estimate the most likely topology. Support for the resulting nodes in the trees were obtained with 1000 (MP) or 200 (ML) bootstrap replicates. The between-group variation in haplotypes (corrected for within-group variation) was calculated using the net nucleotide divergence (D_a) between groups (Kimura 2-parameter model), and the mean pairwise divergence (uncorrected 'p' distances) was calculated within clades or populations using MEGA Version 2.1 (Kumar *et al*. 2001).

Microsatellite analyses

In total, 111 grayling from 4 sampling locations were included in the microsatellite analyses (Fig. 1, Table 1). Based on our previous results (Koskinen *et al*. 2002b) and initial screening of a small number of individuals from each population for PCR amplification success and quality at 17 microsatellite loci, the following 10 markers were selected for further genotyping: *BFRO004* (Snoj *et al*. 1999), *BFRO009* (Sušnik *et al*. 1999a), *BFRO010* (Sušnik *et al*. 2000), *BFRO013* (unpublished), *BFRO015*, *BFRO016*, *BFRO018* (Sušnik *et al*. 1999b), *One2* (Scribner *et al*. 1996), *Str73INRA* (Estoup *et al*. 1993) and *Str85INRA* (Presa & Guyomard 1996). The details of PCR and electrophoresis (on ABI-377) methods of the microsatellites are outlined in Koskinen & Primmer (2001).

Exact probability tests for deviations from Hardy–Weinberg equilibrium (HWE) across populations (within loci) and loci (within populations), exact tests for deviations from genotypic linkage equilibrium (LE) across populations and tests for genetic differentiation among populations were performed using program GENEPOP Version 3.2a (Raymond & Rousset 1995). Corrections for multiple significance tests were performed using a sequential Bonferroni-type correction (Rice 1989). Estimates of population differentiation, F_{ST} (Wright 1951), and their 95% confidence intervals were obtained using the variance component approach (providing the F_{ST} estimator θ; Weir & Cockerham 1984) and bootstrapping of loci, as implemented in FSTAT Version 2.9.3.1 (Goudet 1995).

Genetic relationships between individual grayling were estimated based on the proportion of microsatellite alleles that the specimens shared at each locus, i.e. D_{AS} distances (Bowcock *et al*. 1994). The distance matrix was applied to construct a neighbour-joining phylogram using a program kindly provided by Jean-Marie Cornuet and Sylvain Piry (INRA, Montpellier, France). The D_{AS} matrix was also used in a correlation analysis with the matrix of Euclidean distances based on the meristic data. For this analysis, only 70 specimens could be included, as an individuals' identity to match the genetic and meristic matrices was not available for the remaining grayling. Both simple and partial Mantel tests were used to evaluate the correlation between meristic and genetic distances and the significance of Mantel's *r* was evaluated with 9999 permutations.

To further investigate the distinctiveness of the populations, the accuracy with which individuals could be correctly assigned to their population of origin was assessed using GENECLASS Version 1.0.02 (Cornuet *et al*. 1999). We employed individual assignment tests that utilize a Bayesian statistical approach (Rannala & Mountain 1997), because simulation studies (Cornuet *et al*. 1999), as well as empirical studies in grayling (Koskinen *et al*. 2001), have revealed that the Bayesian method is superior to other procedures implemented in GENECLASS Version 1.0.02. In addition to using the 'direct' assignment test, whereby individuals are always assigned into one of the reference populations, the 'exclusion' test was also applied (Cornuet *et al*. 1999).

Results

Meristic data

Univariate nonparametric tests (Kruskal–Wallis) demonstrated significant differences among all meristic characters except anal rays, but all characters displayed overlapping ranges among the three phenotypic groups, based on minimum and maximum values (Table 2). The phenotype/population groups differed in standard length (SL), and most characters showed some statistical association with length across the entire data set. However, this association was clearly not linear and thus not allometric. For example, eight of the characters for the intermediately sized phenotype are not intermediate in the meristic count (see Table 2). This aspect is seen most clearly for the most pronounced differences such as lateral line pores and total dorsal rays (Table 2). The first two components extracted from PCA explained 40% of the variance in the data and were relatively effective in delineating phenotypes using a bivariate plot (Fig. 2). All individual fish of the lower Amur 'normal' phenotype could be assigned to their own group based on the CDA with a mean probability of 99%. Mean probabilities of correct assignment were also high for orange spot (92%) and the upper Amur population (89%), but there was a low percentage of reciprocal miss-assignment between the upper Amur and orange spot

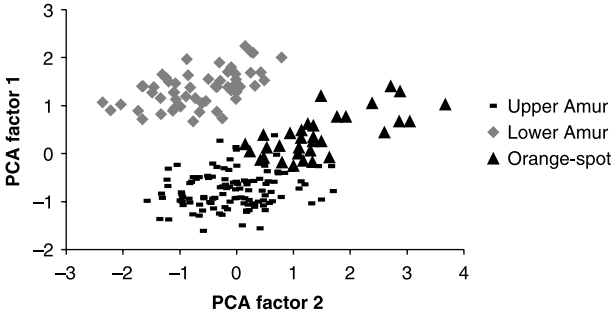

Fig. 2 Scatterplot of the first two principal components derived from 12 meristic characters. Both canonical discriminant analysis (CDA) and individual Euclidean distances used in the Mantel tests used all four PCA factors (with eigenvalues > 1).

groups, as 6 of 117 (5.1%) of the upper Amur individuals were assigned to the orange spot and 3 of 33 (9%) orange spot were assigned to the upper Amur. With the upper Amur population removed from the analysis, the two sympatric forms (orange spot and lower Amur) were assigned to their respective groups with 100% probability.

mtDNA

The complete control region (≈ 1002 bp), 87 bp of tRNA proline, and 11 bp of tRNA phenylalanine were analysed. A total of 33 haplotypes among the 45 individuals were found. There were 85 parsimony informative sites, including ten 1 bp insertions or deletions (indels). Of the 56 sequence evolution models tested using the model test program, the Hasegawa-Kishino-Yano model (HKY) (Hasegawa *et al*. 1985) with a discrete approximation of gamma distribution ($\alpha = 0.0162$) was used for the ML analysis, and the most likely tree found is shown in Fig. 3. For the MP analysis, we found 80 most parsimonious trees. As there were no nodes in conflict between the 50% bootstrap consensus trees derived from MP and ML analyses, the MP tree is not shown and the bootstrap values of the MP analysis have been overlaid onto the ML tree (see Fig. 3). The tree shows three monophyletic clades representing the three phenotype–population groups sampled, i.e. 'orange spot', 'upper Amur' and 'lower Amur' (Fig. 3). Pairwise distances (uncorrected 'p' distances) between haplotypes ranged from 0.012 to 0.057, and the mean genetic divergence between

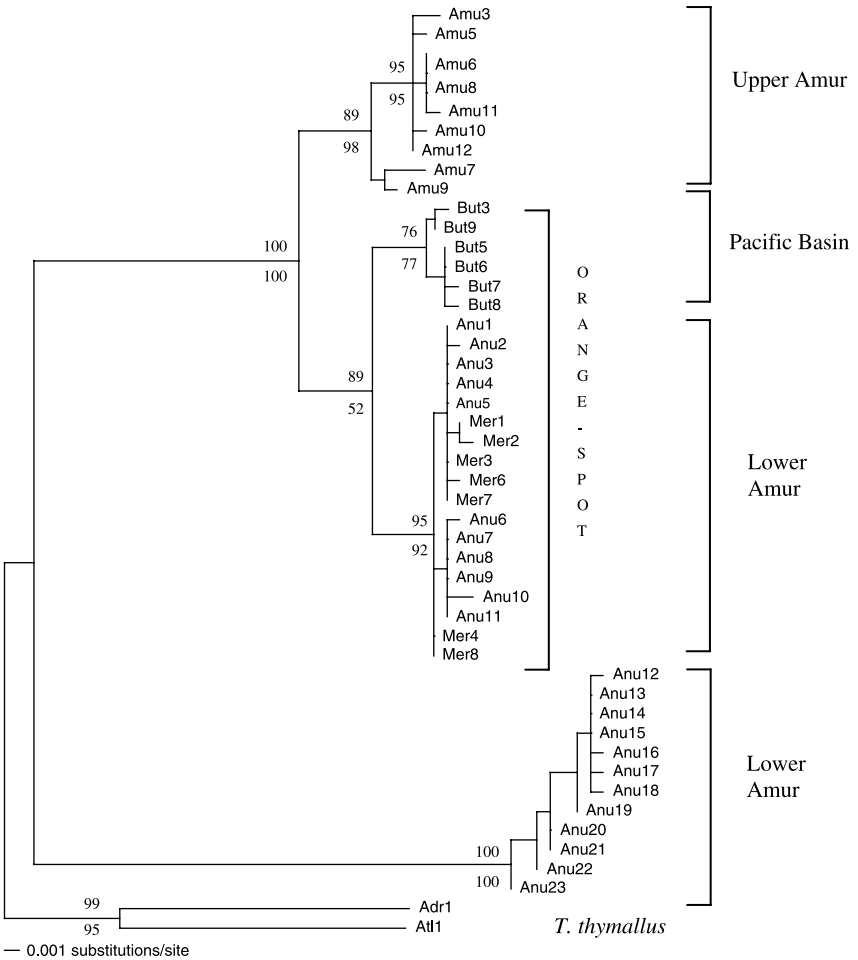

Fig. 3 Maximum likelihood (ML) tree for *Thymallus* based on mtDNA control region and flanking tRNA sequences based on HKY+G model. Bootstrap support values for ML are shown above the branches, and for maximum parsimony below the branches. For population names and codes see Table 1.

Table 3 Pairwise divergence (uncorrected P-distances) between groups corrected for within group diversity with SD in parentheses (upper numbers), the maximum pairwise divergence between groups (lower numbers), and the within group variation (final column) based on the mtDNA sequences. The lower Amur and Orange spot (lower Amur) groups are in sympatry

	Upper Amur	Lower Amur	Orange spot (Buta)	Orange spot (lower Amur)	Distances within groups
Upper Amur					0.003 (0.001)
Lower Amur	0.051 (0.007)				
	0.057				0.002 (0.001)
Orange spot	0.014 (0.003)	0.046 (0.006)			0.002 (0.001)
(Buta)	0.018	0.049			
Orange spot	0.016 (0.004)	0.046 (0.007)	0.008 (0.002)		0.002 (0.001)
(lower Amur)	0.021	0.052	0.012		
Thymallus thymallus	0.028 (0.005)	0.033 (0.005)	0.029 (0.005)	0.031 (0.005)	0.036 (0.005)
	0.049	0.052	0.051	0.052	

the three major clades ranged from 1.4% between the orange spot and upper Amur groups, to 4.6% between the orange spot and the lower Amur 'normal' phenotype (Table 3). Within the orange spot clade, there were two sister sub-clades with 0.8% divergence distinguishing individuals from the Buta River from those of the Amur drainage (see Fig. 1, Table 3).

An additional 74 sampled individuals from the Lower Amur were screened for two diagnostic restriction enzyme sites [5′-GGTGA(N)$_8$^-3′ (*Hph*I) and 5′-G^GNCC-3′ (*Sau*96I)]. These sites were chosen because they revealed bi-allelic polymorphisms that were fixed in the two sympatric phenotypes in the lower Amur (the reactions were carried out in 10 μL volumes for 3 h at 37 °C–5 μL PCR; 4.1 μL H_2O; 0.75 μL buffer; 0.15 μL enzyme). All 74 individuals from the lower Amur screened (20 orange spot; 54 'normal') could be assigned to their phenotype demonstrating that the sites were indeed diagnostic and strengthening the supposition of reproductive isolation between the two sympatric groups.

Microsatellite results

All 10 microsatellites were unambiguously scorable across all of the populations. The exact HWE tests did not reveal any deviations within populations across loci, even before correcting for multiple tests ($P \geq 0.192$). One locus (*BFRO015*) exhibited a significant deviation from HWE when analysed across populations ($P = 0.017$); however, the deviation was insignificant ($P > 0.05$) after correcting for multiple tests. A test of HWE across all loci and populations was not significant ($P = 0.11$). Two instances of significant ($P < 0.05$) deviations from LE were observed in a total of 45 between-locus comparisons across populations. However, neither of these comparisons remained significant after correcting for multiple tests. Genetic differentiation among populations was highly significant ($P < 0.001$). The level of divergence among populations was high,

Table 4 Genetic differentiation based on 10 microsatellite loci, θ_{ST} (95% confidence intervals), among the grayling populations. The estimates are not indicated for the Buta River populations owing to their small sample sizes

	Lower Amur	Orange spot
Lower Amur		
orange spot	0.344 (0.209–0.486)	
Upper Amur	0.280 (0.167–0.400)	0.291 (0.202–0.385)

with θ_{ST} estimates ranging from 0.280 (95% CI = 0.167–0.400) to 0.344 (95% CI = 0.209–0.486; Table 4).

Strikingly, all of the lower Amur, upper Amur and individuals exhibiting the orange spot phenotype clustered into distinct groups in the NJ tree of individuals (Fig. 4). Furthermore, specimens originating from the Buta River formed a subgroup within the orange spot cluster (Fig. 4). Accordingly, the individual assignment tests revealed 100% assignment success of individuals into their correct population of origin. All individuals, except one, could be confidently ($P < 0.05$) excluded from all alternate reference populations. The single exception involved an individual originating from the Merek population that could not be excluded from the Annui orange spot population. This was most likely due to the small sample size (unrepresentative allele frequencies) of the Merek population ($N = 7$).

Microsatellite based D_{AS} distances among individuals were highly correlated with distances based on meristic data (Mantel's $r = 0.382$, $P = 0.001$) for the global data set, as well as when controlling for correlation (partial Mantel test) between the three phenotypic groups (Mantel's $r = 0.339$, $P = 0.001$).

Discussion

All three data sets, encompassing phenotypic and meristic data, as well as maternally and bi-parentally inherited

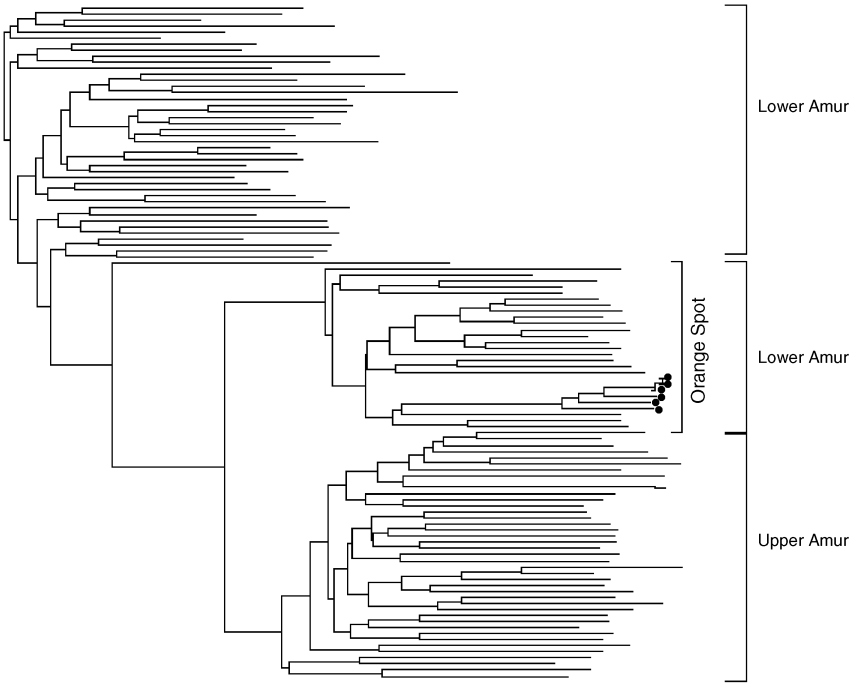

Fig. 4 Neighbour-joining phylogram based on individual microsatellite allele sharing (D_{AS}) values for 111 grayling, sampled across 4 populations. All phenotypic forms clustered together, as indicated, so individual sample number are not included. One symbol (●) is given to represent Buta River (Pacific drainage) 'orange spot' individuals.

genetic markers support the existence of three diagnosable, reciprocally monophyletic and most probably reproductively isolated lineages of grayling within the Amur drainage. As these lineages were previously described as a single species (*Thymallus grubii*), or even a single subspecies (*T. arcticus grubii*), the results are somewhat surprising. The pattern and depth of genetic diversity among these forms also supports a complex phylogeographical history, at least in the lower Amur, probably stemming from major palaeohydrological shifts in drainage patterns during Pleistocene glaciations (Grosswald 1998).

The two sympatric lineages in the lower Amur, in particular, with a mean control region sequence divergence of 4.6%, strongly suggest an allopatric origin with secondary contact and complete reproductive isolation. That the orange spot phenotype also exists in the Pacific draining Buta River, whose headwaters are in proximity to tributaries of the Annui River, underscores the dynamic nature of the hydrological systems in this region. We presume a monophyletic origin for the orange spot phenotype, and thus the ancestors of the Buta River and Annui basin populations must have, at one time, existed in the same drainage basin. Assuming a 1% per Myr divergence rate for the complete mitochondrial control region in *Thymallus*, as reported with an independent calibration in Koskinen *et al.* (2002b) (see also Smith 1992; discussion in Weiss *et al.* 2002), the orange spot lineage could have arisen in the mid-Pleistocene (≈ 0.8 Ma). Further, there is strong bootstrap support (100%) for sister group status of upper Amur grayling and the orange spot lineage (1.4–1.6% divergent) supporting a split in the early Pleistocene (1.4–1.6 Ma) but still much later than the split between orange spot and their sympatric congeners, which display over twice the mean divergence (4.6%) and thus correspond to a pre-Pleistocene common ancestor. Such deep divergence in sympatry is rare among phlyogeographical studies of widely distributed freshwater fishes (Avise 2000). This supports the existence of major historical fragmentation events of the present-day Amur River. Although further sampling in the middle reaches of the Amur basin will be necessary to hypothesize a phylogeographical scenario, its seems plausible that the basin has undergone multiple events of hydrological fragmentation and re-union of tributaries, promoting allopatric speciation in *Thymallus*. This hypothesis is also supported by a relatively recent report of multiple phenotypes in the Bureya River, another Amur tributary in which a distinct form (big-scaled grayling) has been ostensibly described (Antonov 2000) in a basin for which a major river capture event has been independently hypothesized (Grosswald 1988).

In full concordance with the mtDNA sequence data, analysis of 10 microsatellite loci showed a high level of divergence among populations (Table 4), and an unambiguous separation of the three major groups based on the tree of individuals (Fig. 4). At a finer scale, the microsatellite data also supported the distinction of the allopatric Buta individuals, with respect to the remaining orange spot population of the Annui basin. Most importantly, the biparentally inherited microsatellite loci strongly supported complete reproductive isolation between the two sympatric phenotypes (orange spot and lower Amur) with no evidence of hybridization.

Whereas there is often some discordance between nuclear and mtDNA genes in terms of inferring phylogeographical patterns, this study, as well as Koskinen *et al*. (2002b), reveal rather striking concordance across large geographical areas in Asia. Divergence of populations based on microsatellite analysis in European grayling Koskinen *et al*. (2002c) also showed a high level of concordance with that based on mtDNA sequence data (Koskinen *et al*. 2000) suggesting a general pattern within the genus. Considering the depth of the divergence, as well as significant fine-scale population structure displayed in grayling studies thus far (see also Gross *et al*. 2001 and Sušnik *et al*. 2001), it appears that there are some inherent mechanisms promoting population isolation. This hypothesis is strengthened by the comparative work of Froufe *et al*. (2003), which demonstrates stark differences in the divergence of *Thymallus* lineages among east Asian basins compared with three other co-occurring salmonid taxa.

In addition to the single phenotypic character (the orange spot), which is apparently diagnostic for one lineage of Amur basin grayling, phenotypic characterization using meristic characters was useful in delineating all three phenotypic/population groups. The use of individual distances for both the meristic and microsatellite matrices also allowed the demonstration of significant correlation between the two independent data sets, even within the major lineages, supporting at least some genetic basis for the variation in phenotype. Further knowledge of their ecological differences as well as a more extensive survey of the Amur basin where allopatric populations of the two forms may exist is needed to develop working hypotheses on the evolutionary causes of these sympatric lineages. Further, their relation to the populations of the upper Amur cannot be satisfactorily described based on samples 1000 km or more apart, as in this study. As there are no known migratory barriers on the main channel of the Amur, and grayling occur in tributaries throughout its length, investigating populations of the middle reaches of the Amur should uncover contact zones between orange spot and nonorange spot phenotypes. The investigation of these zones may or may not reveal further reproductively isolated groups.

Significance of grayling diversity

Despite a general lack of recognition historically, in comparison with other salmonid genera, it is becoming increasingly apparent that *Thymallus* display a great deal of concordant phenotypic and genetic variation. For example, maximum pairwise sequence divergence in this study, stemming from samples within the Amur basin only, reaches 5.7%, whereas 5.8% is reported for the entire genus of *Salvelinus* (Brunner *et al*. 2001), 2.3% for the polytypic brown trout (Apostolidis *et al*. 1997), and 1.8% for rainbow trout (*Oncorhynchus mykiss*) in western North America where several subspecies were previously recognized (McCusker *et al*. 2000). As all samples within the Amur have been considered by at least some authors to belong to the *T. arcticus* complex, it is also noteworthy that continentally disjunct populations of Arctic grayling were shown to be far less differentiated (0.5–2.4% in the mtDNA control region) (Redenbach & Taylor 1999) than the sympatric populations in the Amur. *T. arcticus* was already shown to be paraphyletic with respect to the Mongolian grayling (*T. brevirostris*) (Koskinen *et al*. 2002b), and thus the genus is clearly in need of systematic revision. This suggestion parallels the promotion of the taxonomic distinction of Adriatic drainage grayling, from *T. thymallus* in Europe (Sušnik *et al*. 2001).

Thus, the genus *Thymallus* appears to be at least as diverse, if not more so, than other groups of salmonids, and the Amur basin in particular may be a biodiversity hotspot with yet-to-be described species. In this study, diagnostic characters, reciprocal monophyly of mtDNA sequences data, and no sign of gene flow or hybridization based on 10 nuclear markers reveal three well-supported taxa within the Amur basin alone. As *T. grubii* was first described at the specific level from the upper Amur basin, this species holds priority to retain its original name reflecting 'Amur grayling'. The lower Amur grayling reported here, living in sympatry with the orange spot phenotype most clearly deserves a species status, whereas it would be presumptuous to propose the same for the orange spot phenotype owing both to its much closer relation with upper Amur grayling populations, and the incomplete knowledge on its distribution and potential for interbreeding with other upper Amur River populations. Clearly, the Amur basin potentially harbours much undescribed diversity, even within a group of fishes like salmonids that are generally considered to be very well studied. We also point out the value of investigating diversity with multiple marker systems and phenotypic data in providing a relatively unambiguous description of biological diversity in the framework of a single investigation.

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