Phylogeography and Population Genetic Structure of Amur Grayling *Thymallus grubii* in the Amur Basin

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ABSTRACT: Amur grayling, *Thymallus grubii,* is an important economic cold freshwater fish originally found in the Amur basin. Currently, suffering from loss of habitat and shrinking population size, *T. grubii* is restricted to the mountain river branches of the Amur basin. In order to assess the genetic diversity, population genetic structure and infer the evolutionary history within the species, we analysised the whole mitochondrial DNA control region (CR) of 95 individuals from 10 rivers in China, as well as 12 individuals from Ingoda/Onon and Bureya River throughout its distribution area. A total of 64 variable sites were observed and 45 haplotypes were identified excluding sites with gaps/missing data. Phylogenetic analysis was able to confidently predict two subclade topologies well supported by maximum-parsimony and Bayesian methods. However, basal branching patterns cannot be unambiguously estimated. Haplotypes from the mitochondrial clades displayed local homogeneity, implying a strong population structure within *T. grubii*. Analysis of molecular variance detected significant differences among the different geographical rivers, suggesting that *T. grubii* in each river should be managed and conserved separately. (**Key Words:** Amur Grayling, Population Genetic Structure, Phylogeography, Mitochondrial DNA Control Region)

INTRODUCTION

Dispersal ability is a key demographic force shaping natural populations and maintaining evolutionary potential (Proctor et al., 2004). Species with limited dispersal abilities display clearer population genetic structure but lower genetic diversity than do species with a tendency towards greater dispersal (Kark et al., 1999; Garcia-Ramos et al., 1999; Bailey et al., 2007). The populations of freshwater fish species from different river systems often show significant genetic differentiation resulting from restricted gene flow, and also there is evidence that some species show high levels of genetic differentiation between populations within a river system (McGlashan et al., 2000). Consequently, changes in interconnectivity within and between drainages have greatly influenced the distribution of many fish species and their genetic structure.

Northeast China has a special and old complex animal fauna and high biodiversity. Based on the geographic distribution, origin and evolution of fishes, there are six

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zoogeographical faunal complexes in this area (Nicolsigy, 1960). In this region, Da xing an Mountain, Xiao xing an Mountain and Chang bai Mountain surround the Northeast Plain, and the five main rivers that flow through this area, Amur River, Sungari, Nenjiang, Yalu and Tumen River, have been repeatedly connected and disconnected with each other during the past million years. It has been proposed that the geological history and topographical complexity of this region would have an effect on species diversity. However, little research has focused on this area, and there is no phylogeographic studies on terrestrial vertebrates or freshwater species in this region.

Amur grayling described at the specific level as *Thymallus grubii* (Dybowski, 1869), have been maintained to support capture and commercial fishing in some temperate countries in the northern hemisphere, and have recently become the focus of conservation efforts in the North Pacific (Mikko et al., 2002). In China, Amur grayling is also a specific and economically important cold freshwater fish with extreme site fidelity and low dispersal capacity. It has only been recorded in the Amur basin and limited to the upper levels of some rivers, which makes the species an ideal model for the study of palaeoclimatic effects on population genetic structure and phylogeography. A recent study of *T. grubii* showed the existence of three

diagnosable, reciprocally monophyletic, and most probably reproductively isolated lineages of grayling within the Amur drainage (Froufe et al., 2003), In this study, therefore, we examine the phylogeography of *T. grubii* and explore the possible mechanisms responsible for the current genetic pattern and to provide an insight into the historical legacy of zoogeographical faunal in Northeast China.

MATERIALS AND METHODS

Amur grayling (N = 95) were collected in 2004-2008 from 10 localities of ten rivers distributed throughout the species' range in the Amur basin in China (Figure 1, Table 1). Based on a comment by Antonov et al. (1996), fish was first noted as to whether they had an obvious yellow-orange ocellus-like spot on the posterior edge of the dorsal fin, and to eliminate the possibility that these samples came from related individuals, we selected many patches from one location. After, a small fin clip had been preserved in 96% ethanol, whole fish were stored at -20 degrees. We also retrieved an additional 12 individual sequences collected from throughout its distribution area in the Ingoda/Onon and Bureya Rivers from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) for evolutionary analyses. (Accession no. AY168390~AY168399, AY779010, AY779009)

Mitochondrial DNA analysis

Total genomic DNA was extracted from the tissue samples using phenol-chloroform extraction (Taggart et al., 1992). The DNA precipitate was re-suspended in TE buffer then stored at -20°C. The complete mitochondrial DNA (mtDNA) control region (904 base pairs) and 200 base pairs of flanking tRNA gene regions were amplified and
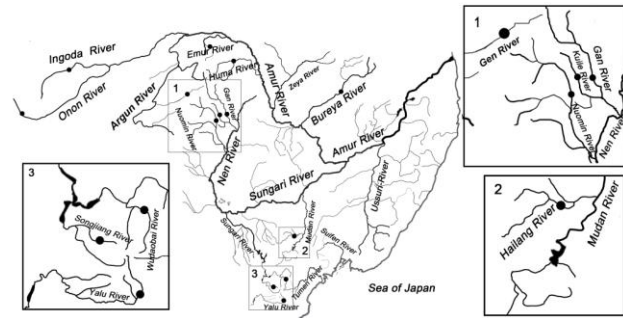
Figure 1. Sampling locations for Amur graying *Thymallus grubii* across whole sampling range and detailed view of sampling sites with 1, 2 and 3.

sequenced. The primers were described in Uiblein et al. (2001). Amplification was performed in a total volume of 50 μl containing 25 mM $MgCl_2$, 10 mM Tris-HCl, 1.5 mM Mg^{2+}, 200 μmol of each dNTP, 0.2 μmol of each primer, 2.0 U Hot start Taq DNA polymerase (QIAGEN), and ≤10 ng of genomic DNA. Thirty cycles were amplified on a Perkin-Elmer Cetus 9700 DNA thermocycler with pre denaturing at 94°C for 3 min; denaturing at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 45 s; and a final 8 min of extension at 72°C. Positive (with DNA template) and negative (with water) controls were used to check PCR performance and contamination. The PCR products were purified using PCR purification Kit (Shanghai Bio-Tec, Ltd) and sequenced with the ABI PRISM BigDye Terminator Ready Reaction Kit (Applied Biosystems) and run on an ABI 377 genetic analyzer according to manufacturer's protocol. To avoid the errors in sequencing, PCR amplifications of all samples were sequenced for both strands.

Table 1. Sample locations including major river basin, geographical coordinates and the number of individuals analyzed for mtDNA variation

Map no.	Population	Basin	Pop code	No. of individuals	Latitude	Longitude
1	Emur River	Amur	EM	4	52° 55′	122° 47′
2	Huma River	Amur	HM	16	52° 18′	124° 42′
3	Ingoda River*	Amur	Amu	10	51° 20′	113° 26′
	Onon River*				48° 75′	110° 25′
4	Gen River	Amur	GE	4	50° 46′	121° 31′
5	Nuomin River	Nen→Sungari→Amur	NM	3	48° 36′	124° 07′
6	Kuile	Gan→Nen→Sungari→Amur	K	3	49° 32′	124° 27′
7	Gan River	Nen→Sungari→Amur	GH	18	49° 42′	124° 35′
8	Hailang River	Mudan→Sungari→Amur	HL	19	44° 28′	128° 54′
9	Yalu River	Yalu	YL	12	41° 26′	128° 12′
10	Songjiang River	deuto-Sungari→Sungari→Amur	SJ	8	42° 10′	127° 30′
11	Wudaobai River	deuto-Sungari→Sungari→Amur	WD	8	42° 16′	128° 25′
12	Bureya*	Amur	Bur	2	51° 55′	134° 53′

* Data is from Froufe et al. (2003a) and Froufe et al. (2003b).

Data analysis

Sequences were aligned by using the CLUSTALX software (1.83) and visually rechecked. Haplotypes were identified using DNASP (Rozas, 2003). Pairwise sequence differences among the haplotypes were calculated using MEGA 2.1 (Kumar et al., 2001). For phylogenetic analysis, we performed maximum-parsimony (MP) using the program PAUP 4.0 (Swofford 2001) and MRBAYES 3.0 (Huelsenbeck and Ronquist, 2001). Modeltest 3.06 (Posada and Crandall, 1998) was run to determine the appropriate model of molecular evolution in a likelihood ratio test framework, and *Thymallus thymallus* and *T. tugarinae* were used as out-groups. Gaps were treated as missing in parsimony analyses. Bootstrap analyses were performed with 6,000 replicates and 1,000 full heuristic replicates for maximum parsimony. For Bayesian phylogenetic inference, four Markov chain Monte Carlo (MCMC) were run for 100,000 generations, sampling every 10 generations; the initial 5% of trees were discarded as burn-in, finally, a 50% majority rule consensus tree was constructed.

Genetic diversity within populations was estimated by haplotype (h) and nucleotide diversities (π) (Nei, 1986) using DNASP (Rozas et al., 2003). Hierarchical analysis of molecular variance (AMOVA) (Excoffier et al., 1992) was performed to compare levels of genetic diversity within and among several possible population groupings of Amur grayling using Arlequin 2.0 (Schneider et al., 2000) with 1,000 permutations. The groupings that maximize values of $\emptyset_{CT}$ and are statistically significant indicate the most parsimonious geographical subdivisions. Genetic differentiation between geographical subdivisions was assessed by comparing average numbers of pairwise differences between distribution ranges (PiXY); average number of pairwise differences within populations (PiX and PiY); and the corrected average pairwise difference (PiXY-(PiX+PiY)/2) using Arlequin 2.0.

To test the hypothesis of demographic expansion, we used the population parameter of the program Arlequin 2.0, to compare the observed distribution of pairwise nucleotide difference among haplotypes within Amur grayling with the expectations of a sudden expansion model (Rogers and Harpending, 1992; Rogers, 1995). For populations experiencing long-term demographic stability, the stochastic process of lineage extinction via genetic drift produces a ragged multimodal distribution. Conversely, a unimodal Poisson mismatch distribution is predicted for populations that have experienced a recent demographic expansion.

Tests for departures from a neutral model of evolution are used widely to infer past demographic expansions or contractions (Ramos-Onsins and Rozas, 2002). Under a demographic expansion hypothesis, the observed pattern of sequence evolution is expected to be significantly different from that predicted under assumptions of selective neutrality and demographic equilibrium. Because we were interested in discriminating between demographic expansion and contraction, we chose two statistical tests, each with particular sensitivity to one demographic scenario. Fu and Li's D* is designed to detect an excess of old mutations, characteristic of a population that has experienced a historical reduction in effective population size (Fu, 1997). In contrast, Fu's Fs is sensitive to an excess of recent mutations, a pattern typical of both a demographic expansion and a selective sweep (Fu, 1997; Ramos-Onsins and Rozas, 2002). D* was calculated in DNASP, Fs in Arlequin 2.0.

RESULTS

mtDNA

From 1,104 bp CR mtDNA sequence generated from 107 individuals, 64 variable nucleotide sites were resolved, including transversion (tv), transition (ts) and 16 insertion/deletions, and 45 haplotypes were defined (Accession no. JF810859~JF810882, EU513355, EU513357~EU513363, EU513365~EU513368, EU513370~EU513372, AY779010, AY168391, AY168397, AY168394, AY168399, AY168396). The H81uf+I+G model was identified using MODELTEST as the best fitting distance estimator, with a gamma distribution shape parameter of 0.7304, a transition/transversion (Ti/Tv) ratio of 1.8181, and base frequencies of A = 0.3281, C = 0.2108, G = 0.1460, T = 0.3181. Parsimony analyses were performed under equal weight (Ti/Vi = 1) and unequal weight (Ti/Vi = 1.8181) sets. All phylogenetic analyses resulted in almost identical tree topologies, and there was geographical structure among haplotypes related to different rivers (Figure 2), which was supported by network analysis (Figure 3).

Forty-five different haplotypes were identified in the 107 samples analyzed (Tables 2 and 3). The number of haplotypes ranged from 1 to 11 for each river. The populations with the greatest number of haplotypes were the Huma River population (11). The haplotype frequency distribution was strongly skewed, with the vast majority of haplotypes found only once (42 out of 45) and restricted to a single population. H44 and H28 were the most common haplotypes and were found in three sampled populations.

Population genetic structure and phylogeography

Diversity indices (average±standard deviation), h and π, are summarized in Table 4. The total of h was 0.972±0.050 ranging from 0.733±0.108 in the Sungari River to 0.924 ±0.075 in the Amur River, and π was 0.0081±0.0004 ranging from 0.0012±0.0013 to 0.0048±0.005 in Sungari and Amur Rivers. Even when there were few samples from some rivers, the results showed a high haplotype diversity and a low nucleotide diversity, and that the genetic diversity
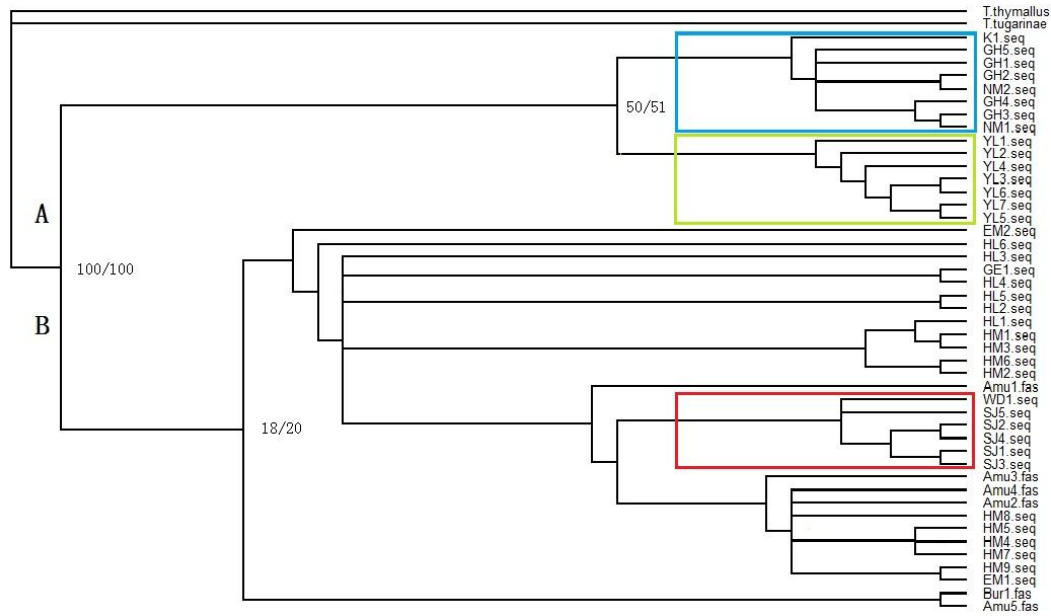
Figure 2. Phylogenetic tree obtained in Bayesian inference from the analysis of D-loop sequences with *Thymallus thymallus* and *T. tugarinae* as out-groups.

for the upper level of Amur (Ingoda/Onon, Huma, Emur and Gen Rivers) was higher than others (Table 4). Haplotypes defined by mtDNA displayed very strong geographical specificity, consistent with river clustering based on different rivers: of the 45 haplotypes, only 4 were shared among rivers. Haplotype 05 was found in both Kuile River and Gen River, Haplotype 39 was found in both Huma River and Gen River Haplotype 28, shared by 7 individuals, was found in 3 rivers (Emur, Huma, Gen Rivers), Haplotype 44, shared by 7 individuals, was found

in 3 rivers (Hailang, Huma, Ingoda/Onon Rivers) (Table 3).

Phylogenetic analysis was able to confidently predict two subclade topologies and was well supported by bootstrap analysis, but basal branching patterns couldn't be unambiguously estimated. In network, we did not find the ancestry of the haplotypes (Figures 2 and 3). Thus, we suggest that there was no obvious phylogeographic pattern within Amur grayling. Pairwise F_{ST} comparisons test among 12 populations showed that the differences were irrelevant to their distributions (Table 5).
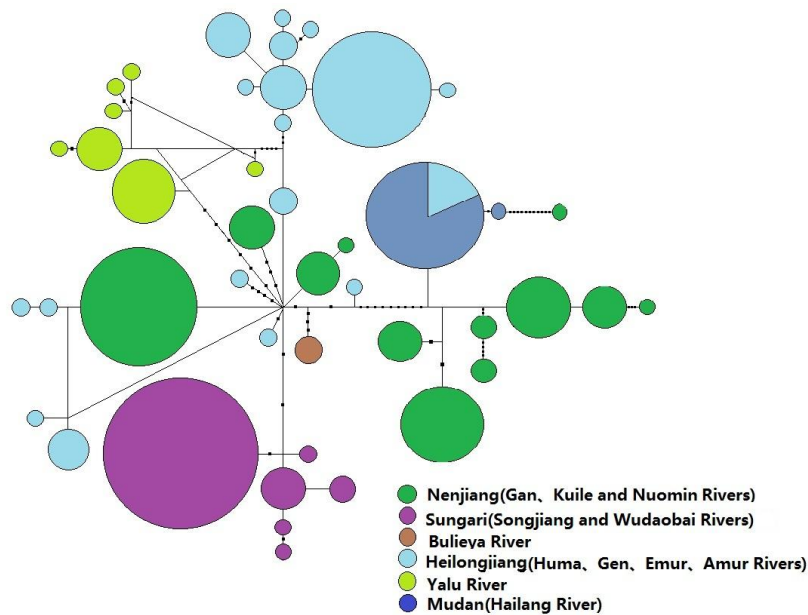
Figure 3. Parsimony haplotype networks for Amur graying. Haplotypes are colour coded based on sampling locality in the map. Circles are sized in proportion to the number of individuals with that haplotype.

Table 2. 64 variable sites of the D-loop region in Amur grayling without considering the alignment gaps. Dots (.) indicate nucleotide identity

```
            [                                             11111111111]
Haplotypes  [     111111111111222233344445555566666667777789999990000000000]
            [     246777700223678889934581382567456782235688114892233782345555678]
            [     624034513160102350249832837612791414539209161146256260290123660068]
Hap_1       ACGTGTTAGTCGGTGGTTACCTGTTGGAGCCTATTCTTAGTCAAGATGTTTGCCTATAACCCAT
Hap_2       .T..........................C......................A...........C
Hap_3       .T..A.A.....................C......................A...........C
Hap_4       ...............CGT.........GCT.C..C...C.....GT.AAC..A.........C
Hap_5       ..................................................A........T..TC
Hap_6       ............................C......................A...........C
Hap_7       ..................................................A........T..C
Hap_8       .TA............CGT.........G.T....C..C......GT.AAC..A...........C
Hap_9       .T.............CGT.........G.T....C..C......GT.AAC..A.....T.....C
Hap_10      .T.............CGT......A..G.T..G.C..C......GC.AAC..A...........C
Hap_11      ..............................................................C
Hap_12      ...A...T.......G.........A...T...AC...........A..AAA....TT....C
Hap_13      .T............CG.........A.G.T...C..C......G..AAC..AT..........C
Hap_14      ..............CGT.........G.T....C..C......GT.AAC..A...........C
Hap_15      G........A..A.C.......G.........A...T...CAC.......A.A.C..A..C...C
Hap_16      ..........A...A......G....A.A.G.TT.AC.............A.A.C..A..C...C
Hap_17      ..............CGT.......A.G.T...C..C......G..A.C..A...........C
Hap_18      ..........A.....A..G.T....A.G.T...C.........T.A.A.CAAA....A....C
Hap_19      ..........A.....G..C.CA.G.T....C..C..T..A.A.C..A..C........C
Hap_20      ..........C.....G..C..CA.G.T...C.....C.T.A.A.C..A..C.......C
Hap_21      ...A......C.....G..C..A.G.T....C.....C.T.A.A.C..A..C........C
Hap_22      ..........A...A......G....A.G.TT.AC.............A.A.C..A..C...C
Hap_23      ..........A.....G...C.A.G.T....C.......TT.A.A.CAAA.........C
Hap_24      ..........C.A...G..C..A.G.T....C........T.A.A.C..A.TC......C
Hap_25      ..........A.A..A......G....A...T...C..........A.A.C..A..C...C
Hap_26      ..........AC....G.........A.G.T...C........AGA.C..A..C.......C
Hap_27      ..........A......A..G....A.G.T...C........T.A.A.CAAA......T.C
Hap_28      ..........A.....G...C.A.G.T....C........T.A.A.CAAA.........C
Hap_29      ..........A.....G...A.G.T....C......AT.A.A.CAAA..........C
Hap_30      ..........C.....G..C..CA.G.T...C........T.A.A.C..A..C.......C
Hap_31      ..........A......A..G....A.G.T...C........T.A.A.CAAA.........C
Hap_32      ..........C.....G..C..A.G.T....C........T.A.A.C..A..C.......C
Hap_33      ..........AA.A..G.........A.G.T...C......A.A.C..A..CT.T......C
Hap_34      .T........A.....G.........A.G.T...C........T.A.A.CAAA.........C
Hap_35      ..........A...A...G.........A.G.T...C........A.A.C..A..C....G.C
Hap_36      ..........A......G.G.....A.G.T...C........T.A.A.CAAA.........C
Hap_37      ..........A.....G.........ATG.T...C..........A.A.C..A..C..T...C
Hap_38      ..........A...A...G.........A.G.T...C........A.A.C..A..C.......C
Hap_39      ..........A.....G.........A.G.T...C........T.A.A.CAAA.........C
Hap_40      ..........A.....G.........A.G.T...C..........A.A.C..A..C..TT....C
Hap_41      ..........A.....G.........ATG.T...C..........A.A.C..A..C.......C
Hap_42      ..........A......A..G....A.G.T...C..........A.A.C..A..C......C
Hap_43      .T........A.....G.........A.G.T...C..........A.A.C..A..C.......C
Hap_44      ..........A.....G.........A.G.T...C..........A.A.C..A..C.......C
Hap_45      ..........A.....G.........A.G.T...AC........A.A.C..A..C.......C
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AMOVA analysis revealed that there were significant subdivisions within populations and among basins of Amur grayling and significant genetic variance was also found among populations, 12.01% of the genetic variance was found among populations (Table 6), which provided evidences that the high level of population structure was related to the hydrological pattern.

Demographic analysis

Values for Fu and Li's D* and Fu's Fs in Amur grayling populations from Amur, Nenjiang, Sungari, Yalu and Mudan Rivers appeared negative but not significant (Table 7), indicating that Amur grayling populations in the Amur drainage were in a process of dynamic balance. The shape of the mismatch distribution for Amur graying was a ragged multimodal curve in the populations from these rivers, which supports the above result.

DISCUSSION

Phylogenetic analysis

Molecular based investigations of *T. grubii* in the Amur basin continued to reveal extensive genetic variation. The Maximum Parsimony tree depicted two highly distinct clades (100% bootstrap support), marked A and B respectively, one representing Yalu and Nenjiang Rivers (A), and the other representing other branch rivers of Amur (B) (Figure 2, MP tree shown). Bayesian method analyses also resulted in almost identical tree topologies, and different populations from Amur River were distinct haplotype groups but with low bootstrap support (<50%). There was geographical structure among haplotypes related to different rivers (Figure 2), which was supported by network analysis (Figure 3).

There existed deep divergence between *T. grubii* in the Nenjiang and Yalu Rivers (Clade A) and *T. grubii* found in the remaining Amur drainage populations (Clade B). Nenjiang River was the source of the Liao River from the Pliocene to the Pleistocene of Quaternary, southward into Bo Sea, reaching to Zeya River basin within the borders of the former Soviet Union. In middle Early Pleistocene of Quaternary, Xiaoxing an Mountain emerged and became the watershed of Amur and Sungari River, separating Nenjiang River and Zeya River. Later, at the end of Early Pleistocene or the start of Middle Pleistocene, SongLiao watershed emerged, which separated Nenjiang River and Liao River.

Table 3. Summary of mtDNA control region haplotype distributions

Haplotypes \ Location	Yalu R..	Huma R.	Wudaobai R..	Songjiang R..	Kuile R..	Nuomin R..	Gan R	Emur R..	Hailang R..	Ing/Ono R.	Bureya R.	Gen R	Total
F01							2						2
F02							4						4
F03						1							1
F04	1												1
F05					2		1						3
F06							5						5
F07							6						6
F08	1												1
F09	1												1
F10	1												1
F11						2							2
F12					1								1
F13	1												1
F14	3												3
F15											2		2
F16		1											1
F17	4												4
F18		1											1
F19				2									2
F20				1									1
F21				1									1
F22		1											1
F23		1											1
F24				1									1
F25										1			1
F26									3				3
F27		1											1
F28		2						3				2	7
F29										3			3
F30				3									3
F31		2											2
F32			8										8
F33												1	1
F34										1			1
F35		1											1
F36										1			1
F37									1				1
F38		3											3
F39		2										1	3
F40									1				1
F41									3				3
F42								1					1
F43										2			2
F44		1							4	2			7
F45									7				7
Total	12	16	8	8	3	3	18	4	19	10	2	4	107

At the end of Middle Pleistocene, the Nenjiang River was forced to u-turn and change its route eastward to flow to the Sungari River, and then into Amur (Liu, 1958; Berg, 1972). Yalu River is not one of the rivers of the Amur drainage; there exists a geographical distance between these two drainages, which is supported by the greater genetic difference between populations from the Yalu River and Amur drainages. Moreover, since the Sungari River and Yalu River both originate in the Changbai Mountain, we inferred that the Yalu River once connected with the Amur drainage (such as Nenjiang River). Maybe later a geographic event such as the formation of Changbai Mountain caused the Yalu River and the Amur drainage to disconnect from each other causing the Yalu River population to split from the Sungari River population. F_{ST} values also indicate that there was no correlation between genetic differentiation and geographic distance.

The pattern and depth of genetic diversity among these forms support a complex phylogeographical history, probably stemming from major palaeohydrological shifts in drainage patterns during Pleistocene glaciations (Grosswald, 1998). The sympatric lineages in Northeast China, in

Table 4. Descriptive statistics of Amur grayling based on control region sequence data

Population	N	H	h	S	K	π
Sungari river	16	6	0.733±0.108	6	1.341	0.0012±0.0013
Wudaobai river	8	1	0.000±0.000	0	0.000	0.0000±0.0000
Songjiang river	8	5	0.857±0.108	6	2.036	0.0019±0.0013
Nenjiang river	24	9	0.869±0.035	21	3.315	0.0035±0.0009
Gan river	18	5	0.791±0.052	16	4.739	0.0043±0.0025
Nuomin river	3	2	0.667±0.314	5	3.333	0.0030±0.0026
Kuile river	3	2	0.667±0.314	16	10.667	0.0097±0.0076
Mudan river	19	6	0.807±0.059	7	1.988	0.0018±0.0003
Hailang river	19	6	0.807±0.059	10	3.088	0.0028±0.0017
Yalu river	12	7	0.864±0.079	11	3.303	0.0040±0.0024
Amur river	34	22	0.924±0.075	28	4.869	0.0048±0.0050
Huma river	16	11	0.950±0.036	17	5.108	0.0046±0.0027
Emur	4	2	0.500±0.265	7	3.500	0.0032±0.0024
Ingoda/Onon river	10	6	0.889±0.075	12	4.889	0.0045±0.0027
Gen river	4	3	0.833±0.222	10	5.167	0.0047±0.0034
Bureya river	2	1	0.000±0.000	0	0.000	0.0000±0.0000
Entire requion (all samples)	107	45	0.972±0.005	64	8.789	0.0081±0.0004

N = Number of individuals; H = Number of haplotypes; S = Number of segregating sites; h = Gene diversity (±Standard deviation); K = Average number of differences; π = Nucleotide diversity (±Standard deviation).

particular, with a mean control region sequence divergence of 12.01%, strongly suggested an allopatric origin with secondary contact and complete geographic isolation. Such deep divergence in sympatry is rare among phylogeographical studies of widely distributed freshwater fishes (Avise, 2000). This supports the existence of major historical fragmentation events of the present-day Amur River (Froufe, 2002). It seems plausible that the basin has undergone multiple events of hydrological fragmentation and re-union of tributaries, promoting allopatric speciation in *Thymallus*.

From the haplotypes shared between rivers, except Amur River with Mandan River and Amur River with

Nenjiang River, we didn't detect gene flow between different populations. In the reduplicative climate changes, species range shrunk, and species retreated to one or more shelters (Broccoli and Manabe, 1992; Hewitt, 1996). Huma River had the most haplotype diversity. We infer that perhaps the Huma River was the shelter area for Amur grayling when the palaeoclimate changed. Our data supports a relatively recent hydrological mixing of major river drainage systems in the Amur basin, as shared haplotypes had already been demonstrated.

Population genetic structure and phylogeography

Our results revealed low nucleotide diversity (π =

Table 5. Pairwise FST values among Amur grayling populations

	Gan	Nuomin	Kuile	Yalu	Bureya	Huma	Amur	Emur	Gen	Songjiang	Wudaobai	Hailang
Gan	0.0000											
Nuomin	0.2874*	0.00000										
Kuile	0.2927*	0.3824	0.0000									
Yalu	0.7482*	0.7422*	0.6891*	0.0000								
Bureya	0.7863*	0.8778	0.6547	0.7556*	0.0000							
Huma	0.7330*	0.7147*	0.6500*	0.6197*	0.5665*	0.0000						
Amur	0.7203*	0.7208*	0.6151*	0.6181*	0.5635*	0.0514	0.0000					
Emur	0.7506*	0.7888*	0.6339*	0.6667*	0.7519	0.0262	0.0979	0.0000				
Gen	0.7416*	0.7368*	0.5863*	0.6434*	0.6551*	0.0108	0.0650	-0.1556	0.0000			
Songjiang	0.8028*	0.8666*	0.7821*	0.7428*	0.8375*	0.4810*	0.5057*	0.6554*	0.6014*	0.0000		
Wudaobai	0.8282*	0.9550*	0.8628*	0.7943*	1.0000*	0.5439*	0.5980*	0.8331*	0.7642*	0.6122*	0.0000	
Hailang	0.7630*	0.7974*	0.7293*	0.6853*	0.6578*	0.2916*	0.2508*	0.4791*	0.4443*	0.5838*	0.6557*	0.0000

Asterisks indicate significant values after Bonferroni correction. Amur represents Onon and Ingoda Rivers.

Table 6. The results of AMOVA for Amur grayling D-loop estimated using Φ-statistics

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	Φ-statistics (p-value)
Among basins	4	348.589	3.65684	57.22	Φ_{ST}: 0.69227* (p = 0.000)
Among populations wthin basins	7	46.667	0.76759	12.01	Φ_{SC}: 0.28072* (p = 0.000)
Within populations	95	186.838	1.96672	30.77	Φ_{CT} : 0.57217* (p = 0.000)
Total	106	582.093.243	6.39114		

* $p<0.01$.

0.0081±0.0004) and high haplotype diversity (h = 0.972±0.005) in Amur grayling (Table 4), which could reflect a short evolutionary history of the population. This scenario was also supported by the different branching among haplotypes in the phylogenetic tree and the "star-like" shaped network (Figures 2 and 3) as well as demographic history analysis. There was a high proportion of genetic variation between different rivers of the Amur basin (Φ_{SC}: 0.28072*), which suggested there was significant genetic differentiation within Amur grayling populations, with few haplotypes shared among rivers. This unique genetic structure was related to the evolution history and biological characteristics of Amur grayling. Amur grayling have unique life history characteristics, such as the limited dispersal abilities and a strong retracing of river spawning (Northcote, 1995; Nykänen et al., 2005; Gönczi 2008). These biological characteristics easily led to familial populations and limited the level of gene flow between populations and resulted in significant genetic variation between geographical populations. Moreover, habitat fragmentation could also cause genetic differences between different populations. The result of the nested clade analysis also indicated there may exist habitat fragmentation and restricted gene flow in the history of the Amur grayling population.

Significant fine-scale population structure in Amur grayling was found, and it appeared that there were some genetic mechanisms promoting population isolation (Gross et al., 2001; Sušnik et al., 2001), which is strengthened by the comparative work of Froufe et al. (2003). Our MSN network showed that haplotypes were geographically structured while tests of AMOVA indicated that 57.22% of the genetic variance was due to between basin differences, and 30.77% within population variation, which was further

supported by significant evidence of genetic differentiation from F_{ST} statistics. Only 12.01% of the genetic variance was due to among populations within basins.

The haplotypes distribution of Amur grayling displayed distinctly regional concentration, haplotypes from the mitochondrial clades showed a river related genetic structure among all populations sampled across Amur basin except the individuals from Emur and Huma Rivers and significant genetic differentiation between each of these populations. These results were also reflected in the distribution of haplotypes in the MSN network, in which the haplotypes formed a river related network. Bayesian clustering of genotypes also revealed clear subdivision except for the Amur populations.

Mismatch distribution and the no significant Fu and Li's D and Fu's Fs values indicated that population dynamics of Amur graying were complicated. We couldn't explain this process with only one demographic expansion or contraction. On account of changes in interconnectivity within and between drainages and effects of paleoclimate, Amur graying experienced complicated history dynamic, but each population appeared dynamic balance.

Conservation and management strategy of Amur grayling

Although the genetic diversity level of Amur grayling populations is high, fishery resources face endangered status, so conservation and management work is urgently required. The significant differences of Amur grayling between Nenjiang River, Yalu River and other populations in the Amur drainage indicates a retained low gene flow. According to the result of AMOVA and the unique life history characteristic of grayling, it is necessary to treat populations in Nenjiang, Yalu River, Sungari River and

Table 7. Fu and Li's D* and Fu's Fs test for control region sequences

	Heilongjiang R	Mudan R	Sungari R	Nenjiang R	Yalu R
D	-1.295	-0.566	-0.416	-1.531	-0.718
Fs	-14.55	-3.51	-3.78	-3.33	-3.18

Asterisks indicate significant values, $p<0.01$.

Amur River as different so that conservation management units can conserve the peculiar genetic diversity. So far, the decrease of the habitat and human activities are the main factors threating Amur grayling populations rather than the decrease of genetic diversity. Therefore, we suggest the work of conservation focuses on restoring and protecting the habitat in future.

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