

## European grayling phylogeographic lineages of Russian European North from barcoding DNA fragment

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### Abstract

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MtDNA *COI* barcoding fragment was analyzed in 302 European graylings (*Thymallus thymallus* L.) from 38 localities of the Russian European North. Four *T. arcticus* Pallas haplotypes were found in 62 fishes from the rivers of the Kola Peninsula and 3 fishes from the Northern Dvina basin; that indicates an ancient introgressive hybridization of European and Arctic graylings. Two *T. thymallus* haplotypes differing by two substitutions dominated in most of studied fishes and formed two haplogroups, one of which dominates in most water bodies of the Kola Peninsula, Pechora and some tributaries of the Northern Dvina. In the northeast, another haplogroup dominated and might be diagnostic for the identification of Russian European North European grayling. The observed distribution of haplotypes reflects post-glacial colonization and is associated with paleoclimatic changes that fragmented the grayling range.

Thus, *COI* barcoding fragment can be used to identify origin of grayling and is suitable for describing phylogeographic lineages and indicating evolutionary significant units (ESU).

**Keywords:** European grayling; *T. thymallus* L.; arctic grayling; *T. arcticus* Pallas; introgressive hybridization; *COI*; Russian European North

### Introduction

The areal of European grayling, *Thymallus thymallus* (L.), covers most of Europe, extending from Britain to the Urals and from waterbodies of Barents Sea basin to the waterbodies of Mediterranean Sea basin (Berg, 1948; Kottelat & Freyhof, 2007). In a number of countries, European grayling is an object of hatchery-rearing and reintroducing. European grayling, notable for its high taste qualities, is a pop-

ular object of recreational fishing, and the state of grayling populations is of wide interest to the general public (thus, European grayling has been several times declared the “fish of the year” in Austria and Germany). It should be noted that the habitation of grayling in a waterbody can be considered as an indicator of the temperature and purity of water and, in a sense, the preservation or disappearance of grayling in a particular waterbody is a widely noticeable indicator of environment situation.

In general, the European grayling is not characterized as a threatened species, being included in the “Least Concern” IUCN Red List category (Freyhof, 2013), but in a number of countries and regions it has an endangered species status and is under protection (Lyach & Remr, 2019; Weiss et al., 2013). It is estimated that the number of European graylings as a whole continues to decline, and the number of weak populations is increasing (Persat, 1996), there has been increased also the number of waterbodies where native European grayling populations have ceased to exist in relatively recent times.

In general, it is recognized that additional targeted efforts are required for preserving grayling populations and grayling populations diversity, and this increases attention to the study of the species ecological and genetic structure and characteristics (Gum et al., 2006; 2009; Weiss et al., 2021). Considering that populations of grayling, a stenohaline and not performing long migrations species, are highly specific in terms of morphological and genetic parameters, genetic study of grayling is important for the formation of sustainable fishing and aquaculture approaches. Development of grayling conservation and management strategies and initiatives demonstrates the trend of transition from efforts to preserve and maintain the species to efforts to preserve and maintain the diversity and uniqueness of the populations of the species (Dawney et al., 2011; Marić et al., 2011; Weiss et al., 2002).

The planning of work on the conservation and management of European grayling populations is increasingly associated with the tendency to revise the previously established views and ideas about the diversity of the species and its position among *Thymallidae* in general. For solving these issues and clarifying the genetic characteristics of the European grayling, it seems necessary to pay special attention to the study of grayling populations that are least affected by economic activity. These populations currently include the grayling populations of waterbodies in the Russian European North.

## Materials and Methods

In total, 302 European graylings from the Russian European North rivers were analyzed. The samples' locations are shown in Figure 1. DNA extraction from fixed with 96% ethanol fins was performed using the QIAGEN DNeasy™ kit (QIAGEN, Germany) according to the manufacturer's protocol. To amplify *COI* gene region universal *COI* primers for fish were used (Ivanova et al., 2007). PCR was performed in total of 20 µL volume containing 2 µL of 10X AmpliEN buffer, Mg<sup>2+</sup> 2.0 mM, 0.8 mM of each DNTP's, 1U BioHYTaq

DNA polymerase (DIALAT, Russia), 5 pmol of each primer (Syntol, Russia), 1 µL template DNA. The amplification was performed in the following mode: I – 95° C 5 min., then 35 cycles II – 94° C 20 sec., 20 sec. 52° C, 72° C 1 min., III – final elongation 72° C – 10 min. Horizontal electrophoresis in 1.5% agarose gel was conducted for PCR product control. The PCR products were purified by precipitation mix of ammonium acetate and 70% ethanol. BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA), was used for the sequence reaction; the analysis of the nucleotide sequence was carried out in ABI Prism 3500 genetic analyzer (Applied Biosystems, USA).

Nucleotide sequences primary processing and multiple alignment were performed using the Geneious® 6.0.5 software (Biomatters Ltd), the number of haplotypes (H) was determined in FaBox software (Villesen, 2007). Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best, the analysis was performed in MEGA X program (Kumar et al., 2018). The most suitable model was applied to construct haplotypes trees: neighbor joining (NJ) with bootstrap support (10,000 iterations) and maximum likelihood (ML) in the MEGA X program (Kumar et al., 2018). Bayesian tree (chains of 400 000 generations with the first 10% discarded) was built in the MrBayes program plugin (Ronquist et al., 2012) implemented in Geneious® 6.0.5. The MSN (minimum spanning network) algorithm was used to construct haplotype network in the PopArt program (Bandelt et al., 1999).

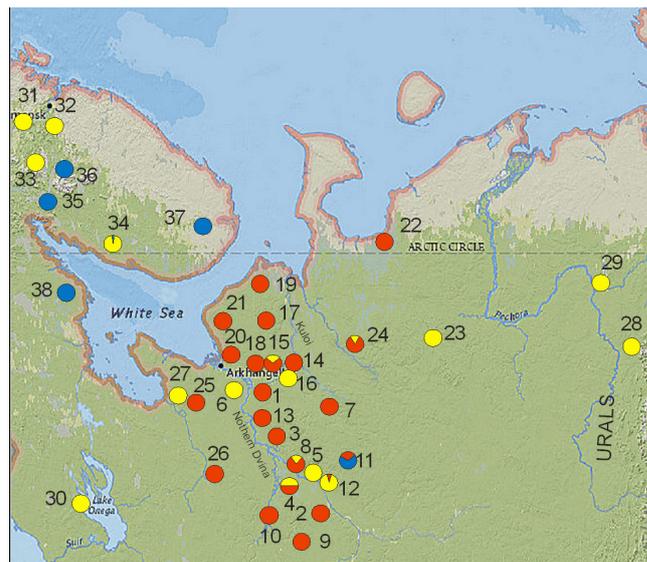


Fig. 1

Table 1

N loca- tion	GenBank number	Haplogroup 1 (Kola-Pechora)						Haplogroup 2 (Northern Russian)			<i>T. arcticus</i> haplotypes				
		OL672530	OL672531	OL672532	OL672533	OL672534	OL672535	OL672536	OL672537	OL672538	OL672539	OL672540	OL672541	OL672542	
	Haplotype	Kola	ND-1	ND-2	ND-3	Pech-1	Pech-2	ND	Ku	One	Umba	ND-Vyya	Värzuga	Ponoy	
		<i>Northern Dvina basin</i>													
1	Chuplega							4							
2	Kestvazh							12							
3	Ladozera							10							
4	Lemen'ga	1		2				1							
5	N. Toima	2													
6	Oboksha				2										
7	Pokshen'ga							2							
8	Topsa	2						7							
9	Uhtanga	4													
10	Ust'ya							1							
11	Vyya							1			1	2			
12	Yorga		5					1							
13	Yula							2							
		<i>Kuloi basin</i>													
14	Kelda							11							
15	Laka	2						15	9						
16	Polta	1						2							
17	Sojana							14							
18	Sotka							1							
19	Megra							13							
20	Mud'uga							3							



The obtained sequences were deposited in GenBank under the accession numbers (OL672530 – OL672542) shown in Table 1. The nucleotide sequences containing the studied mtDNA region deposited in the GenBank were used – FJ853655 (Finland, hatchery), MT063001 (Slovenia), MT063002 (Austria), MT063005 (Rein), MT063045 (Rona), MT06312 (*T. arcticus*, Ob'), MW36549 (*T. brevirostris*) – to construct haplotypes trees and network.

## Results and Discussion

686 bp region of *COI* gene was studied after sequences multiple alignments. A total of 13 haplotypes were identified. Haplotypes were subdivided into 2 groups differing by 23-24 substitutions (~3.3% of differences). Most of the studied sequences (236) belong to the first group of haplotypes (Table 1). 9 variable sites were identified, from them 8 transitions and 1 transversion, all substitutions are synonymous.

The 9 obtained haplotypes were subdivided into 2 more main groups, differing by 2-3 substitutions. 128 fish have haplotype designated by us as ND. The haplotype designated as Kola was dominant (in 85 fish) in the other group (Table 1).

The second group of haplotypes included 65 sequences. The haplotype, designated by us as Uмба, was found in 61

fish. In this group, most of the specimens originated from the waterbodies of the White Sea basin of the Kola Peninsula and three fishes from the Vyva River (the Northern Dvina basin) (Table 1). In this group, 4 haplotypes were identified (Uмба, Ponoj, Varzuga, ND\_Vyva) (Table 1). The sequences differed by one substitution. 3 transitions and 1 transversion were detected, all substitutions were synonymous.

K2P+Y (0.05) transitions model was used to construct the trees. On the Bayesian tree (Figure 2), the haplotypes from the first group were clustered with the haplotypes of the *T. thymallus* with numbers FJ853655 (Finland, hatchery), MT063001 (Slovenia), MT063002 (Austria), MT063005 (Rein), MT063045 (Rona), the second group formed a common cluster with the haplotype of the *T. arcticus* (MT06312) from the Ob' basin, which coincided with the haplotype found in most fish from the second group. In the cluster of European graylings with high support at the node (1.0/80/84), haplotypes Ku, ND and One are distinguished, forming an independent branch (Figure 2).

To build haplotype network, haplotypes belonging to the European grayling cluster were used. The structure of the network is dominated by two haplotypes, forming 2 haplogroups. In the first haplogroup, designated by us as Kola-Pechora, the star-shaped topology is most clearly traced (Figure 3), this

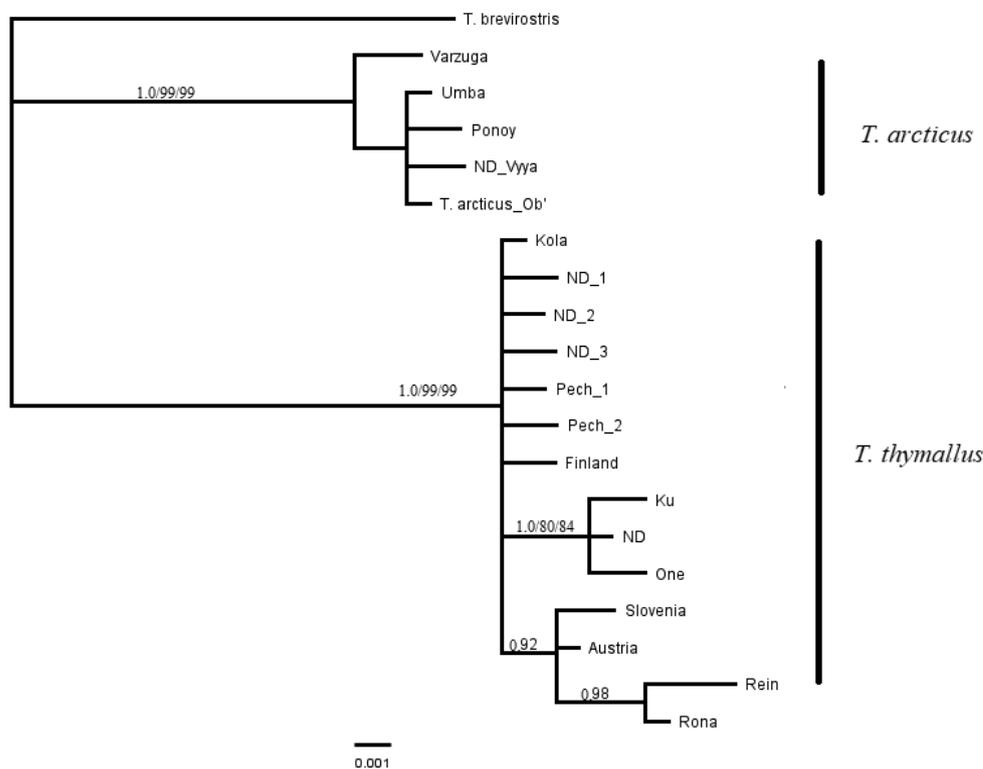


Fig. 2

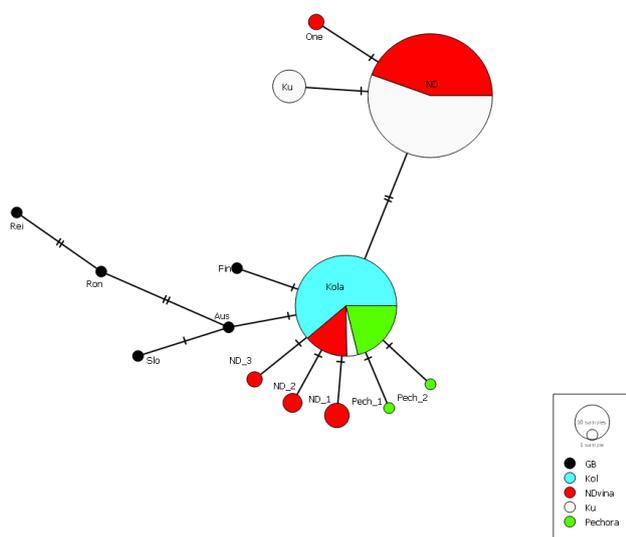


Fig. 3

group includes fish from most of the studied waterbodies of the rivers of the Kola Peninsula, Lake Onega and large rivers of the European North, and the same haplotype, which coincides with sequences from GenBank was found in fishes from the Pechora (MT062995) and the Ural (MT062999) rivers.

In the second haplogroup, designated by us as Northern Russian, there were fishes from the tributaries of the Kuloi, Northern Dvina, Onega, Mezen' and small rivers of the Kuloi plateau coast of the White Sea and the Oma River. For a visual perception of the locations of samples with this haplogroup, the sampling scheme was mapped (Figure 1). In the rivers of the Kola Peninsula, Lake Onega, Pechora, only sequences of the Kola-Pechora haplogroup were found, while the haplotypes of this group were absent in small rivers of the Kuloi plateau coast of the White Sea and the Oma River. The haplotypes of the Northern Russian group dominated in the northern part of the rivers, with the exception of the Pechora.

The range of the European grayling in the east is limited by the Ural Mountains (Berg, 1948; Reshetnikov, 1998) and this species does not occur on the territory of Siberia. The range of Arctic grayling extends from the Ural Mountains along the Arctic coast of Asia and America (Berg, 1948; Reshetnikov, 1998); it is important to note that in some waterbodies on the western side of the Urals this species lives sympatrically with European grayling, for example in the Kara River (Berg, 1948; Zinoviev, 1988) and in a number of tributaries of the Pechora River (Ponomarev, 2014; Zi-

noviev, 1979). In these waterbodies, hybrids of European and Arctic grayling were found, they were well identified by both species' features overlapping, and these hybrids were fertile (Ponomarev, 2014; Zinoviev, 1979; 1988). Also, natural grayling hybrids were studied using isoenzyme markers and there was showed that they form hybrids of at least the second generation (Shubin & Zakharov, 1984; Shubin et al., 2004). The interspecific hybridization phenomenon is quite widespread in fish (Makhrov, 2008; Yamamoto et al. 2006; Zhivotovsky et al., 2016). Recently it has been widely studied with the development of molecular genetic methods (Araujo et al., 2021; Gruzdeva et al., 2018); the number of works on natural hybrids as well as on hybrids appeared as a result of transfer or invasion is increasing (Fukui et al., 2016). Often hybridization processes reflect disadvantageous conditions for one of the species (for example, decreasing in numbers due to overfishing), or the influence of climatic change (Araujo et al., 2021). In addition, by mtDNA analysis, traces of ancient hybridization could be revealed (Radchenko, 2004). In our work, we have for the first time found out traces of ancient hybridization of European and Arctic grayling on the Kola Peninsula, which occurred at a time when the range of Arctic grayling spread further westward than it is now. Also, haplotypes of Arctic grayling were identified by us in the Vyva tributary of the Pinega River – the eastern tributary of the Northern Dvina; earlier, Arctic grayling haplotypes were identified in one of the Pinega tributaries – Syamzhenga River (Koskinen et al., 2000). Pinega flows in karst rocks on the territory of the Kuloi plateau, where a large presence of Siberian-Ural flora and fauna is noted (Saburov, 1972); some species with a break in their range are present in the Urals and on the territory of the Kuloi plateau, for example, plant species: *Salix recurvigemmis*, *Dianthus repens*, *Gypsophila uralensis* Less. subsp. *Pinegensis* (Red Book of the Arkhangelsk Region, 2002). In addition, some Siberian species of animals live here, for which this territory is the northwestern border of the range: *Salamandrella keyserlingii* (species of salamanders), *Cuculus optatus* (species of cuckoos) (Saburov, 1972). There are various hypotheses about the colonization ways of Siberian species into the territory of Europe; they are most fully presented in the review by Makhrov et al., 2020.

In the waterbodies on the territory of the Kuloi plateau, we identified haplotypes of the European grayling, characteristic only for the northeast of the Arkhangelsk region, belonging to the Northern Russian haplogroup. In the work of Skurikhina (2018), presumably in the area of the Mezen' Bay of the White Sea, bordering the Kuloi plateau, a glacial age refugium for Pacific smelt is supposed. So, we can also suppose the existence of a refugium for grayling in the

same region. Thus, genetic characteristics of grayling from the Kuloi plateau waterbodies should be considered in designating Evolutionarily Significant Units (ESU) for European grayling; Kuloi plateau populations could be regarded as ESUs.

## Conclusion

The *COI* fragment proved to be effective for identifying the phylogeographic structure of the European grayling in the European North of Russia. There were revealed traces of the ancient hybridization of *T. arcticus* X *T. thymallus*, which turned out to be much farther west than previously assumed. Arctic grayling haplotypes dominated in the White Sea basin waterbodies of the Kola Peninsula (Umba, Ponoï, Seydozero). In addition, Arctic haplotypes were found in the basin of the Pinega River, the left tributary of the Northern Dvina.

Among the European haplotypes, two haplogroups stand out, one of which dominates in most waterbodies of the Kola Peninsula, Pechora, and southern tributaries of the Northern Dvina. In the northeast, there dominated another haplogroup, differing by two substitutions; it can be diagnostic for the identification of European grayling from this region.

Given that in the history of grayling population management, other *Thymallidae* sometimes were introduced in waterbodies where European grayling lived or lives (Freyhof, 2013), the study of the coexistence of European grayling with other grayling species is also of significant importance for further practical research. The study of unaffected populations is also important for developing a strategy for managing other populations, including populations whose genetic structure is formed as a result of the introduction of fish from other waterbodies, maintenance and restoration of threatened populations.

At the same time, taking into account the connection between the history of grayling settlement and climatic changes, the study of the peculiarities of the species reflecting postglacial dispersal is important for the formation of scenarios of response to climatic changes.

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