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A Comparative Analysis of the Far Eastern Grayling Species *Thymallus tugarinae* and *Thymallus grubii flavomaculatus* Based on the Data from Mitochondrial DNA Cytochrome *b* Gene Sequencing

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Abstract—Nucleotide sequences of a fragment of mitochondrial DNA cytochrome *b* gene were obtained from two species of graylings that inhabit the Russian Far East, viz., *Thymallus tugarinae* and *T. grubii flavomaculatus*. A phylogenetic analysis of four *Thymallus* species, whose relationships have been poorly studied, was performed on these new data as well as on several sequences from the NCBI GenBank sequence database. The rate of genetic divergence between the Far Eastern graylings *Thymallus tugarinae* and *T. g. flavomaculatus* corresponded to that between European (*T. thymallus*) and Arctic (*T. arcticus*) graylings. Moreover, single nucleotide substitutions that result in alterations of the amino-acid structure of protein products (non-synonymic mutations) were revealed between sequences of the cytochrome *b* gene in the Far Eastern grayling species. The topology of phylogenetic trees, which was composed by means of Bayesian analysis and the maximum parsimony method, showed four independent phylogenetic lineages of graylings. In addition, the phylogenetic relationships between *T. tugarinae* and *T. g. flavomaculatus* were supported statistically. The obtained data indicated that Far Eastern graylings *T. tugarinae* and *T. g. flavomaculatus* are distinct species and confirmed that they belong to different phylogenetic lineages, as found earlier.

Keywords: *Thymallus*, cytochrome *b*, Bayesian analysis, phylogeny, graylings
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INTRODUCTION

Graylings are a specific group of fish that inhabit fast-running waters of riverine systems in the boreal zone. The range of their distribution within a river basin extends from the periphery of upper reaches and streams down to the lower course of a river. This is a typically stenobiontic group, which can neither live in slowly flowing and brackish waters nor migrate to neighboring river basins by the sea. The recent studies of graylings were targeted at investigating their taxonomy and reconstructing the historical migration routes of their ancestral forms.

Currently, many on genetic data are dedicated to morphological variability and also to the systematics and phylogeny of graylings [3–5, 7–10, 13–16, 19–25, 27, 30, 32–34, 37–39]. These works mostly discuss problems regarding the taxonomy of some forms of graylings, their distribution and biogeography. One of the controversial issues in grayling systematics is the taxonomical status of the Far Eastern forms, *Thymallus tugarinae* and *T. grubii* (including both subspecies, *T. g. grubii* and the Yellow-spotted Grayling *T. g. flavomaculatus*), which inhabit the Amur River and also several rivers that flow into the Sea of Japan and Sea of Okhotsk. A number of authors consider Amur graylings as a single polyphyletic species with numerous morphological variants [2]. One of the latest works [18] that was dedicated to the reconstruction of the phylogenetic relationship between all the described grayling species, shows differences in the Amur species of the genus *Thymallus* both from one another and from other grayling lineages (*T. thymallus* and the rest of species in the genus *Thymallus*). However, the distinctions that were found are not supported statistically, which does not allow one to consider them as different species.

To solve this problem, we reconstructed the phylogenetic relationships of *T. tugarinae* and *T. g. flavomaculatus*, which are two representatives of the genus Thymallus. The nucleotide sequences of the mitochondrial DNA cytochrome *b* gene were used as a molecular marker. This marker is widely used in estimating the intra- and inter-specific variations in verte-

Table 1. The geographic distribution of populations of the studied graylings and the ID numbers that were assigned to this and the external group of fish in the NCBI GenBank sequence database

Population, species	Basin	Coordinates	*n*	ID No. by GenBank, NCBI
Langry River *Thymallus tugarinae*	Langry → Strait of Tartary → Pacific Ocean	52°78′ N 142°60′ E	13	FR851931–FR851943
Samarga River *Thymallus grubii flavomaculatus*	Samarga → Sea of Japan → Pacific Ocean	47°78′ N 138°29′ E	14	FR851944–FR851957
Fish farm, Rautalampi *Thymallus thymallus*	City of Turku → Baltic Sea → Atlantic Ocean	60°47′ N 22°27′ E	1	FJ853655
Yukon *Thymallus arcticus*	City of Whitehorse → Yukon → Bering Sea → Pacific Ocean	60°71′ N 135°06′ E	1	FJ872559
Thymallus arcticus	Unpublished		1	GQ452036
Oncorhynchus masou			1	AF125210
Salvelinus alpinus			1	AF154851
Coregonus baunti			1	FJ589213
Coregonus chadary			1	FJ589214
Osmerus mordax dentex			1	HQ115272
Clupea harengus			1	EU552606
Engraulis encrasicolus			1	EU552563

brates and reconstructing their phylogenies [1, 11, 12, 17, 33].

MATERIAL AND METHODS

Nucleotide sequences of the mtDNA cytochrome *b* gene of two grayling species that inhabit the Russian Far East, *Thymallus tugarinae* ($n = 13$) and *T. grubii flavomaculatus* ($n = 14$), were obtained in this work. Data on the species *T. arcticus* and *T. thymallus* (Table 1) from the GenBank sequence database (NCBI) were also used.

For the molecular and genetic analysis, we took fragments of cardiac or body muscles of fish that were collected in the Langry River on Sakhalin Island, and the Samarga River in the Primorsky Krai (Table 1). DNA extraction was performed with a Genomic DNA Purification Kit produced by Fermentas according to its protocol. The cytochrome *b* gene was amplified through the polymerase chain reaction (PCR) method using the upstream primer FishcytB-F (5'-ACCAC-CGTTGTTATTCAACTACAAGAAC-3') and the downstream primer THR-Fish2-R (5'-AACCTC-CGACATCCGGCTTACAAGACCG-3') [31]. The amplification was conducted in a Bio-Rad C1000 thermal cycler in 25 ml reaction mixture, including 2.5 μl of 10× Taq polymerase buffer, 2.5 μl of 50 mM $MgCl_2$ solution, 2.5 μl of 2.5 mM primer solution, 20–50 ng of DNA, 0.3 μl of Taq polymerase 5000 units/ml, and 10.5 μl of distilled and deionized water. The PCR was conducted under the following conditions: primary denaturation for 3 min at 94°C in 35 cycles, including denaturation for 30 s at 94°C, primer annealing for 30 s at 57°C, and elongation for 1.5 min at 72°C. The program was completed with an extra elongation for 5 min at 68°C with subsequent cooling to 4°C. The quantity of DNA and PCR products was measured through electrophoresis in an ethidium bromide-stained 1.5% agarose gel, with subsequent visualization in UV light (312 nm).

The sequencing reaction was conducted in a 10 μl mixture, which contained 0.5 mM primer, 10–100 ng of PCR product, and 1.5 μl of BigDye Terminator Cycle Sequencing Kit V.3.1. The reaction was performed according to the protocol for the BigDye Terminator Cycle Sequencing Kit. The products of the sequencing reaction were read in an ABI 3130 × 1 genetic analyzer at the research laboratory of the Cell Biology and Genetics Chair of the Far Eastern Federal University (FEFU).

Assembly and multiple amino-acid alignments were carried out in the CLUSTAL-W program module and integrated into the MEGA 4.0 [26]. In order to find an optimum model for calculating the genetic distances, Modeltest v. 3.7 was used [29]. The GTR

model was selected as the optimum one [36]. The reconstruction of phylogenetic relationships was performed in the PAUP 4b10 [35]. Phylogenetic relationships were reconstructed with the methods of neighbor-joining (**NJ**), maximum parsimony (**MP**), maximum likelihood (**ML**), and Bayesian analysis (**BA**).

RESULTS AND DISCUSSION

The amplified fragment of the mtDNA cytochrome *b* gene included nearly 1100 nucleotide pairs (np). After the assembly and multiple amino-acid alignments, the length of the studied fragment of the gene reached 1017 np. In *Thymallus tugarinae*, there were seven variable sites, and four parsimony-informative sites. In *Thymallus grubii flavomaculatus* there were nine and five sites, respectively. The numbers of variable and parsimony-informative sites of the studied fragment of cytochrome *b* gene in the combined sample of graylings, including both these species, grew significantly to 86 and 80, respectively.

The nucleotide structures of the studied fragment of mtDNA cytochrome *b* gene in various grayling species showed almost no differences, averaging 29.8%, 28.9%, 25.3%, and 16.0%, respectively, for the A, T, C, and G nitrogenous bases. The nucleotide structures of the sequences that were obtained for the first, second, and third codon positions were generally similar in various grayling species. The frequencies of all nitrogenous bases at the first codon position were approximately equal, while the second position featured a higher thymine content (40.9%) and cytosine and adenine prevailed at the third codon position (35.5% and 33.6%, respectively). Moreover, a low guanine content was detected at the third position of codon (6.8%). The frequency of guanine at this position was the most changeable in various species of the genus *Thymallus*, fluctuating within 5.5–8.2%. Proportions of nucleotide contents such as these are typical for both graylings [40] and salmonids [17].

The studied fragments of cytochrome *b* gene in certain grayling species varied in the frequency of codon usage as well. In a number of cases, we observed fixed differences in amino-acid coding. For instance, the triplets CAG, GAG, and UGG that code glutamine, glutamic acid, and tryptophan, respectively, were found in *T. tugarinae*, while they were absent in *T. g. flavomaculatus* (Table 2).

The genetic divergence between sequences of mtDNA cytochrome *b* gene in *T. tugarinae* and *T. g. flavomaculatus* was 7.7%, which matches the interspecific divergence values for this group of fish. When comparing these species to European and Arctic graylings, the divergence level decreased insignificantly ($d = 7.2–7.5\%$) (Table 3).

The Phylogenetic relationship of the studied grayling species were reconstructed based on the data of cytochrome *b* gene sequences of 1017 np in length, including the data of the GenBank sequence database. Sequences of the fragment of cytochrome *b* gene from various fish species in GenBank were used as an outgroup (see Table 1).

As is known, in most animal species, sequences of mtDNA cytochrome *b* gene during pairwise comparison develop characteristic mutations of the third codon position and replacements of the transition type (replacement of monotypic bases), which creates so-called phylogenetic noise and distorts the real pattern of phylogenetic relationships [1, 28]. For this reason we analyzed the obtained cytochrome *b* gene sequences for transition mutations at the third codon position at various taxonomic levels. As a result, it was found that mutations arise during pairwise comparison of higher-rank taxons (for example, when comparing various families) at the 25% level of divergence of sequences (Fig. 1). Because the magnitude of the divergence between the species we studied is lower, we took all types of replacements at every codon position into consideration in the phylogenetic analysis.

Phylogenetic reconstructions for the four studied species generally gave similar tree typologies via various methods used and also showed a high degree of differentiation between various grayling species. Every reconstruction had four classes, which corresponded to four grayling species. In the phylogenetic tree that was reconstructed through the maximum likelihood algorithm, *T. tugarinae* and *T. g. flavomaculatus* proved to be sister species with a sufficient bootstrap support (74%). *T. arcticus* appeared to be the closest to the group *T. tugarinae/T. g. flavomaculatus*, while *T. thymallus* formed a separate basal branch. An analogous topology was obtained through Bayesian modeling (Fig. 2). The likelihood support of the group *T. tugarinae/T. g. flavomaculatus* was even higher (86%).

The maximum parsimony method allowed us to compose one tree of 1031 steps in length with the following values for main indices: CI = 0.64; RI = 0.80; RC = 0.52; HI = 0.41 (Fig. 3). The topology of this tree also revealed four species clusters. However, unlike the *ML* topology, *T. g. flavomaculatus* in this case did not have a common node with *T. tugarinae* and formed a separate branch. The species *T. arcticus* and *T. thymallus* also formed two independent branches and *T. arcticus* proved to be basal one. A topology similar to that at the *MP* was formed by means of the neighbor-joining method. However unlike *MP*, here *T. arcticus* and *T. thymallus* were sister species to one another and formed a basal branch.

These results indicate a significant divergence between the grayling species *T. tugarinae* and *T. g. flavomaculatus* in the Russian Far East, which is comparable to the interspecific level. This is manifested by both the high divergence level and the fixed amino-acid replacements during pairwise comparison of mtDNA cytochrome *b* gene sequences of various species to one another.

Table 2. The codon frequencies (%) of the mt DNA cytochrome *b* gene in two grayling species

Codon	Both species	*Thymallus tugarinae*	*Thymallus grubii flavomaculatus*	Codon	Both species	*Thymallus tugarinae*	*Thymallus grubii flavomaculatus*
UUU(F)	11.5	12.0	11.0	UAU(Y)	4.6	5.0	4.2
UUC(F)	17.6	17.0	18.1	UAC(Y)	9.4	9.0	9.8
UUA(L)	**4.6**	**3.0**	**6.0**	UAA(*)	0.0	0.0	0.0
UUG(L)	2.8	2.6	3.0	UAG(*)	0.0	0.0	0.0
CUU(L)	10.5	11.0	10.0	CAU(H)	1.0	1.0	1.0
CUC(L)	6.0	6.0	6.0	CAC(H)	10.0	10.0	10.0
CUA(L)	23.6	22.0	25.0	CAA(Q)	5.2	4.4	5.9
CUG(L)	**3.4**	**6.0**	**1.0**	**CAG(Q)**	**0.8**	**1.6**	**0.0**
AUU(I)	12.6	11.0	14.0	AAU(N)	5.0	5.0	5.0
AUC(I)	13.9	14.9	13.0	AAC(N)	8.0	8.0	8.0
AUA(I)	7.0	8.1	6.0	AAA(K)	6.0	6.0	6.0
AUG(M)	2.0	1.0	3.0	AAG(K)	1.0	1.0	1.0
GUU(V)	6.4	6.0	6.9	GAU(D)	5.5	5.0	6.0
GUC(V)	5.0	5.0	5.0	GAC(D)	3.6	4.2	3.0
GUA(V)	10.0	10.0	10.0	GAA(E)	4.5	3.9	5.0
GUG(V)	2.0	3.0	1.0	**GAG(E)**	**0.5**	**1.0**	**0.0**
UCU(S)	6.4	7.0	5.9	UGU(C)	0.0	0.0	0.0
UCC(S)	5.0	5.0	5.0	UGC(C)	4.0	4.0	4.0
UCA(S)	5.5	6.0	5.0	UGA(*)	9.5	9.0	10.0
UCG(S)	1.2	0.4	2.0	**UGG(W)**	**0.5**	**1.0**	**0.0**
CCU(P)	**5.4**	**3.8**	**9.0**	CGU(R)	0.0	0.0	0.0
CCC(P)	5.6	6.2	5.0	CGC(R)	1.0	1.0	1.0
CCA(P)	6.3	7.0	5.6	CGA(R)	5.9	6.0	5.7
CCG(P)	**0.7**	**1.0**	**0.4**	**CGG(R)**	**0.1**	**0.0**	**0.3**
ACU(T)	2.6	2.0	3.1	AGU(S)	1.0	1.0	1.0
ACC(T)	10.0	11.0	9.0	AGC(S)	0.0	0.0	0.0
ACA(T)	8.9	7.7	10.0	AGA(R)	0.0	0.0	0.0
ACG(T)	**0.6**	**1.3**	**0.0**	AGG(R)	0.0	0.0	0.0
GCU(A)	**6.4**	**8.0**	**4.9**	GGU(G)	2.6	2.0	3.1
GCC(A)	16.5	16.0	17.0	GGC(G)	5.0	4.9	5.0
GCA(A)	6.0	5.0	7.0	GGA(G)	11.0	11.0	11.1
GCG(A)	1.0	1.0	1.0	GGG(G)	6.4	7.0	5.9

Table 3. The values of the *p*-distance between certain grayling species, %

Species	1	2	3
1. *Thymallus tugarinae*	–	–	–
2. *Thymallus grubii flavomaculatus*	7.7	–	–
3. *Thymallus thymallus*	7.7	7.5	–
4. *Thymallus arcticus*	4.6	4.2	6.0

The work by Froufe et al. [18], which was based on the combined analysis of the controlling region and the ATP6 gene of mtDNA, showed the interrelationship of all currently known grayling species and subspecies. According to these authors, there are four primary phyletic lineages of graylings, which diverged approximately at the same time and subsequently evolved and isolated within 1.6–4.8 million years ago. In particular, according to these data, the Far Eastern species *T. grubii* and *T. tugarinae* were sister species; however, this relationship had low bootstrap support (under 50%). Meanwhile, it is known that the reliably established nodes are those in which the value of bootstrap analysis is higher than 70, while values of 90 of higher are better [6].

The phylogenetic relationship that we reconstructed using the *MP* and *BA* methods almost completely conformed to the data that were obtained earlier on the basis of the combined analysis of sequences in the control region (the *D*-loop) and the ATP6 gene in mtDNA [18]. In our work, the support of phylogenetic relationships of Far Eastern graylings during Bayesian modeling reached 86%, which statistically proves the opinion that was expressed by Froufe et al. [18]. The topology of the phylogenetic reconstruction that was built through maximum parsimony and neighbor joining showed the independent evolutionary history of *T. g. flavomaculatus* and *T. tugarinae* with bootstrap support of 99% (Fig. 3). The level of genetic divergence of clusters that include *T. g. flavomaculatus* and *T. tugarinae*, according to the data obtained by Froufe et al. [18], amounted to 4.8% and 5.3% (respectively, to the data from the sequencing the fragments of the ATP6 gene and the D-loop), which was 2 or 3 times higher than the values of divergence between other clusters. According to our data, the values of the *p*-distances between *T. g. flavomaculatus* and *T. tugarinae*, which were also included in different clusters, insignificantly exceeded the divergence level that was found when the sequences of these two species were compared to European and Arctic graylings (see Table 3). According to E.A. Zinovyev [2], whose opinion rests upon the morphological data of other authors, there are no distinct diagnostic signs by which to differ Amur grayling species (*T. g. flavomaculatus, T. g. grubii, T. tugarinae*, and *T. burejensis*); variations of their morphotype remain at the intraspecific level. Based on both the literature and our own genetic data, we believe that the divergence between Amur grayling

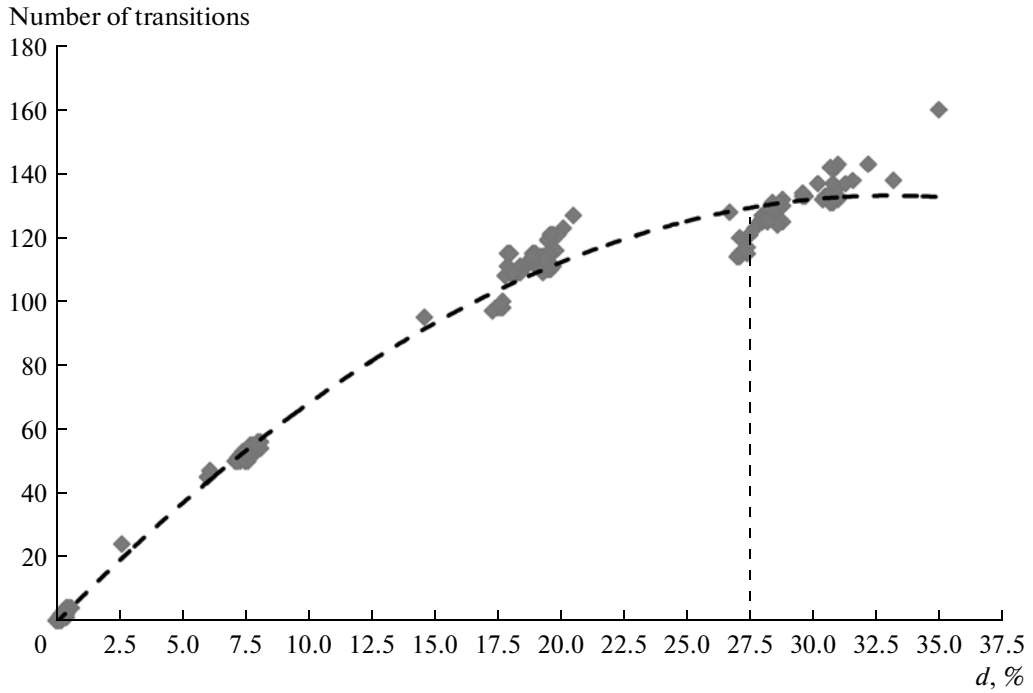

Fig. 1. Results of the analysis of mtDNA cytochrome *b* gene sequences for transition mutations in fish.

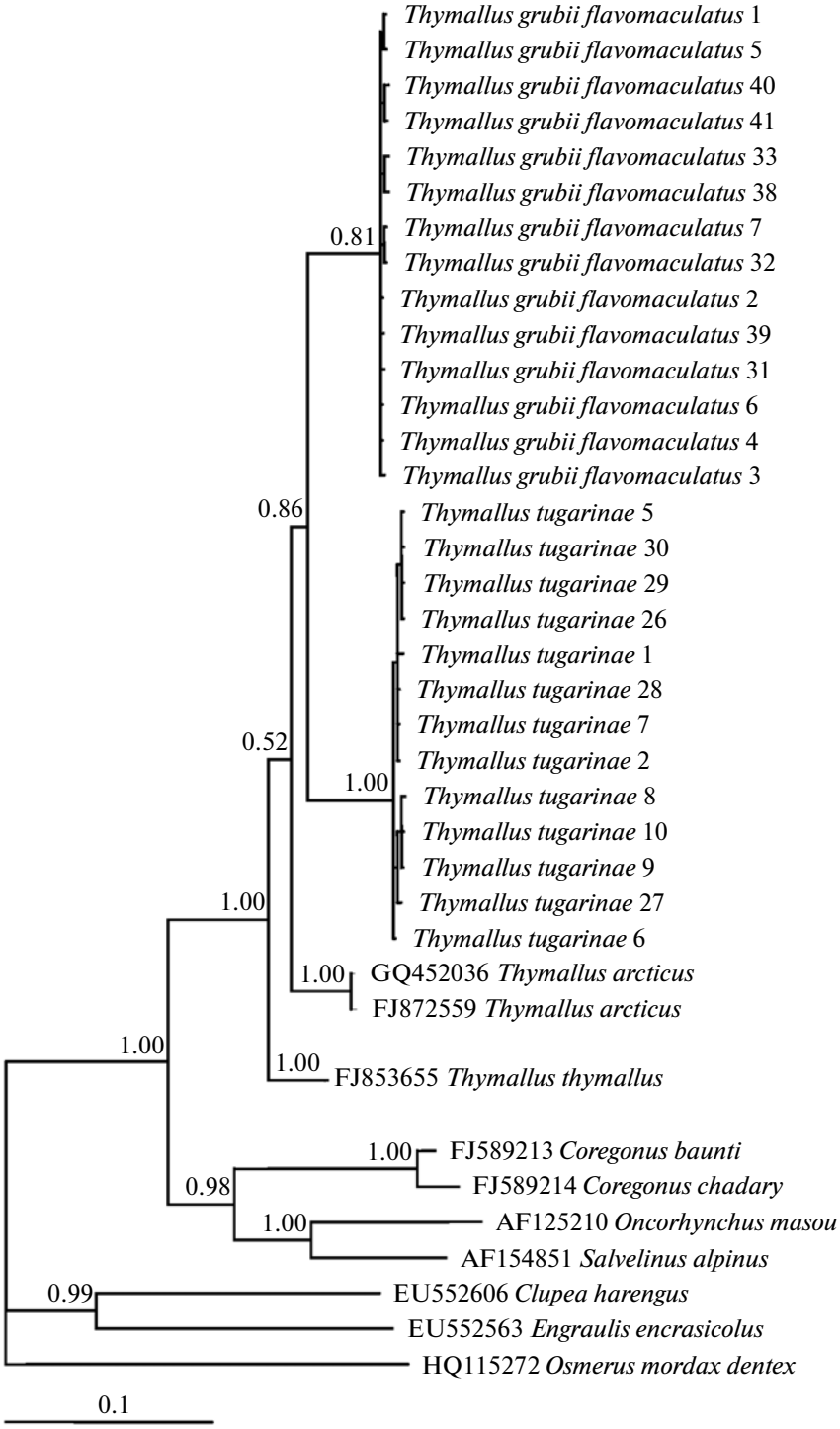

Fig. 2. A phylogenetic tree composed based on the data of mtDNA cytochrome *b* gene sequencing by means of Bayesian modeling (*BA*).

species takes place at a level comparable to species, which is distinguished by morphological signs (for example, the *T. thymallus* and *T. arcticus complex*). A value of the *p* distance equal to 7.7% (Table 3) undoubtedly indicates their independence as species.

The results of phylogenetic analysis based on the data from mtDNA cytochrome *b* gene sequencing agree with the data that were obtained by Froufe et al. [18] on the existence of four main phylogenetic lineages of graylings. Thus, *T. g. flavomaculatus* and *T. tugarinae*

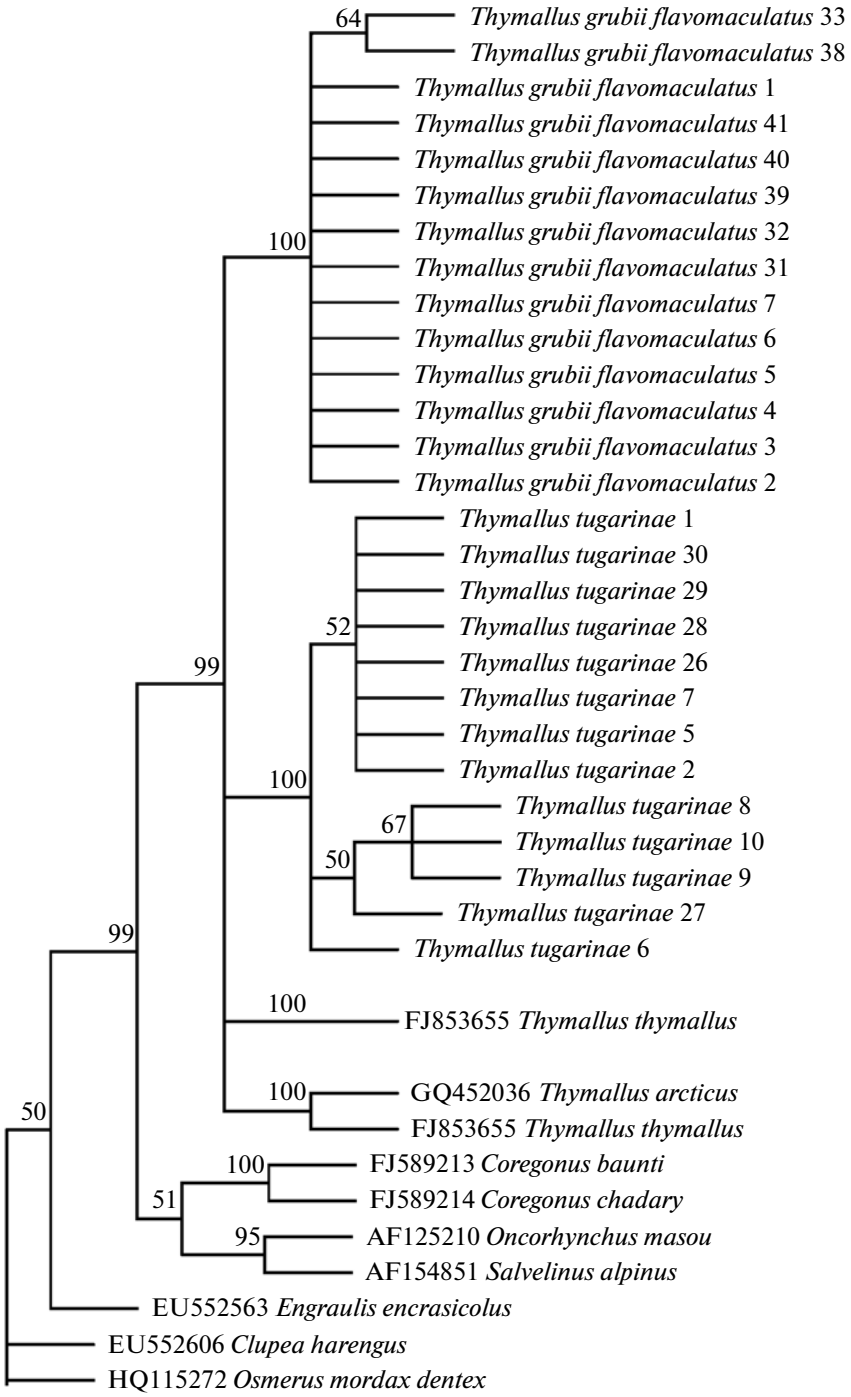

Fig. 3. Phylogenetic tree composed on the data of mtDNA cytochrome *b* gene sequencing with the maximum parsimony method (*MP*).

could probably have diverged a little later than representatives of the remaining phylogenetic lineages did. To solve this problem, it is necessary to use molecular markers of nuclear DNA, which is the subject of our future studies.

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