

## Grouping of Baikal omul *Coregonus autumnalis migratorius* Georgi within the *C. lavaretus* complex confirmed by using a nuclear DNA marker

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Previous studies of the mtDNA cytochrome *b* gene and control region indicated that the Baikal omul was closely related to the Baikal whitefishes, but not to the Arctic cisco, *Coregonus autumnalis* Pallas, its closest taxonomic relative. Moreover, extremely high mtDNA similarity between Baikal omul and Baikal lacustrine whitefish, *C. lavaretus baicalensis* (Dyb.), was revealed. Direct sequencing of PCR-amplified DNA of the first internal transcribed spacer of ribosomal DNA (rDNA ITS1) of five taxa was carried out using previously designed primers to determine the position of the species within the Coregonidae: two ciscoes including the Arctic cisco, *C. autumnalis* Pallas, and the Irish pollan, *C. pollan* Thompson; and three of Lake Baikal's coregonids including Baikal omul, *C. autumnalis migratorius* Georgi, Baikal lacustrine whitefish, *C. lavaretus baicalensis* Dybovsky, and Baikal pidschian, *C. lavaretus pidschian* Pallas. The study showed again that Baikal omul grouped within the *C. lavaretus* complex. The trees that were obtained correlated with our previous results based on mtDNA analysis. We conclude that Baikal omul is one of many representatives of the *C. lavaretus* complex.

### Introduction

The history of studies on the origin and taxonomic status of the Baikal omul, *Coregonus autumnalis migratorius*, is typical of the difficulties that researchers encountered when investigating the evolution of coregonids. The Baikal omul inhabits the pelagic zone of Lake Baikal up to the depths of 350–400 m, and is a typical coregonid

fish with a terminal mouth, a large number of gill rakers, and the body characteristics of fish that feed in the pelagic zone. Due to general morphological similarity, the Baikal omul has been considered a close relative of the Arctic cisco, *C. autumnalis*, for more than two centuries. Because the Baikal omul is completely isolated from the Arctic cisco and has a number of morphological differences (Gašowska 1960, Shaposhnikova

1968), different authors have only considered the problem of how that isolation had appeared and if it was, indeed, a separate species, until recently. However, recent genetic investigations have shown not only the genetic distinctiveness of Baikal omul but also its similarity with the whitefishes. Thus, analyses of allozymes and restriction fragments of mtDNA from six Siberian coregonid species have revealed the affinity of Baikal omul to the Palearctic whitefishes (Politov *et al.* 2000, 2002), and sequencing of the mtDNA fragments of Lake Baikal coregonids have demonstrated close relationships between the Baikal omul and the Baikal whitefishes (Baikal pidschian — *C. lavaretus pidschian*, and Baikal lacustrine whitefish — *C. lavaretus baicalensis* (Dyb); Sukhanova *et al.* 2000, 2002). Moreover, both studies have demonstrated an extremely close resemblance between the mtDNA sequences of Baikal omul and the Baikal lacustrine whitefish (Sukhanova *et al.* 2000, 2002, Politov *et al.* 2000).

Although, in our opinion, the genetic investigations described above demonstrate that the Baikal omul belongs to the *C. lavaretus* complex, the results of allozyme analyses as well as the similarities in mtDNA do not completely exclude a hypothetical hybrid origin for Baikal omul (such as between Arctic cisco and a member of the *C. lavaretus* complex; Politov *et al.* 2000, Sukhanova *et al.* 2002). Thus, for example, Baikal omul has allozyme alleles that unite it with Arctic cisco and distinguish it from the whitefishes (Bodaly *et al.* 1994, Politov *et al.* 2000, 2002). This fact suggests the hypothesis of hybridization. At the same time, the results from the same loci obtained in these works do not coincide completely. Although the allozyme analysis of Bodaly *et al.* (1994) testifies to the significant genetic differentiation between Baikal omul and other forms of Arctic cisco, it unites them in one monophyletic group, while Politov *et al.* (2000, 2001) demonstrated not only the distinctness of Baikal omul, but also its proximity to the whitefishes rather than to Arctic cisco. Significantly, no Baikal whitefishes were included in either study. Because sequencing mtDNA has revealed the monophyletic distinction of Lake Baikal coregonids with respect to all other whitefishes (Sukhanova *et al.* 2002), it

is possible to assume that if these forms had been included in the allozyme analyses, they could have influenced the results that were obtained. Additional independent nuclear DNA markers could help solve the problem.

In this study, we use sequencing of the first internal transcribed spacer of ribosomal DNA (rDNA ITS1) to estimate phylogenetic relationships of the same Lake Baikal coregonids that were analyzed by mtDNA sequencing (Sukhanova *et al.* 2000, 2002). This particular nuclear marker (rDNA ITS1) was chosen since it had already been used successfully in the phylogenetic analysis of other representatives of the genus *Coregonus* (Sajdak & Phillips 1997). We compared the results obtained from the analysis of rDNA ITS1 of Baikal omul, its closest taxonomic relatives, Irish pollan (*C. pollan*) and Arctic cisco in addition to its sympatric neighbours, the Baikal whitefishes, with the results of the previous genetic studies performed by us and other investigators. It allowed us to draw some conclusions with respect to Baikal omul evolution and to discuss some aspects related to phylogenetic relationships within the Coregonidae.

## Materials and methods

The taxa that were studied are listed in Table 1. Total DNA for the polymerase chain reaction (PCR) was isolated from ethanol preserved liver by phenol chloroform extraction according to Sambrook *et al.* (1989). The primers used for PCR, and direct sequencing of PCR-amplified DNA of rDNA ITS1 are described by Sajdak and Phillips (1997). The primers, MD-1 forward and 28S reverse, were used in initial PCR amplifications to produce a DNA fragment spanning the ITS1, ITS2 (second internal transcribed spacer) and 5.8S rDNA. Then, 1  $\mu$ l of the resulting solution that contained this long fragment was added as a template in the amplification of ITS1 using two other primers, KP-2 and 5.8S. Magnesium chloride requirements differed among species and were 1 mM for whitefishes and 1.5 mM  $MgCl_2$  for ciscoes. PCR amplifications were carried out according to Sajdak and Phillips (1997). Double-stranded DNA PCR products were cleaned using QIAquick PCR Purification

Kit protocol (QIAGEN K.K., Japan) before sequencing. Cycle sequencing of PCR-amplified DNA was done using Amersham Dye Terminating sequencing kit and KP1 forward and 5.8S reverse primers from both strands to produce overlapping fragments. An ABI model 373A sequencer was used for electrophoresis (CER, Kyoto University, Japan). Because a length polymorphism involving a repeating unit of 65–67 base pairs (bp) was found in the rDNA ITS1 in the *C. lavaretus* complex (Sajdak & Phillips 1997), we screened additional individuals of *C. lavaretus pidschian* from water bodies adjacent to Lake Baikal for length variation using PCR amplification with the KP-2 forward –5.8S reverse primer set (Table 1).

The previously published ITS1 sequences of other representatives of the Coregonidae (Sajdak & Phillips 1997) were combined with those obtained in this study. In the beginning, the sequences were aligned by the CLUSTAL W

Multiple Sequence Alignment Program (version 1.7) and then corrected by hand.

The MEGA 2.1 program (Kumar *et al.* 2001) was used for the following analyses. The Jukes-Cantor (Jukes & Cantor 1969) and Kimura two-parameter (Kimura 1980) models of DNA substitutions were used for calculation of genetic distances. Phylogenetic trees were constructed using the Neighbour-Joining (Saitou & Nei 1987) and Maximum Parsimony (Fitch 1971) methods. The pair wise deletion of the gaps options was used, and the sequences were bootstrapped 500 times.

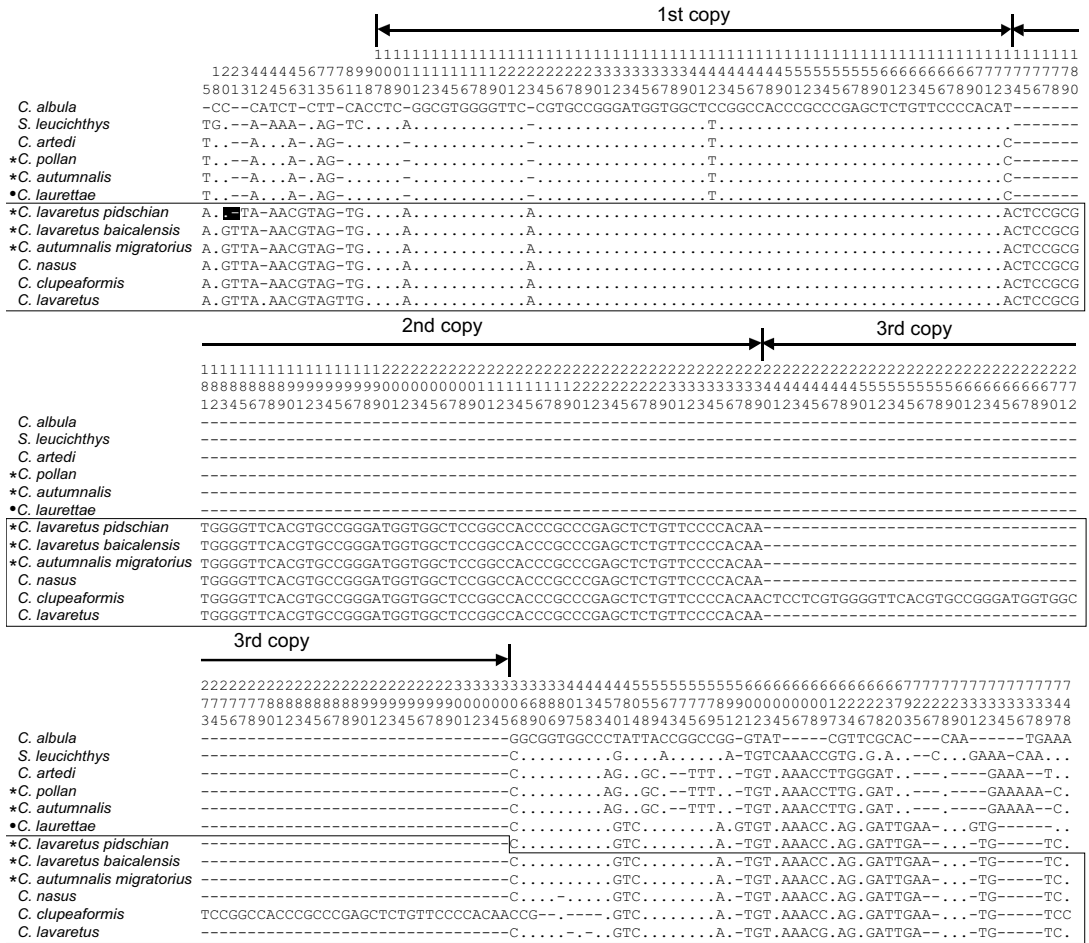
## Results

The nucleotide sequences of ITS1 were obtained for five taxa (Table 1): ciscoes including two specimens of Arctic cisco (593 bp) and two Irish pollan (594 bp); and the Lake Baikal coregonids

**Table 1.** Studied samples of coregonid fishes.

Species	Location	Coordinates	N	Date
Baikal omul ( <i>Coregonus autumnalis migratorius</i> Georgy)				
	Baikal basin:			
	Bargusin River	53°65'N, 109°75'E	10	October 1993
	Maliy Chyvirku River	53°55'N, 109°5'E	2	October 1994
	Lake Kulinda	55°92'N, 109°95'E	2	October 1996
Baikal lacustrine whitefish ( <i>Coregonus lavaretus baicalensis</i> Dybovsky)				
	Baikal basin:			
	Lake Baikal (Chyvirku Bay)	53°60'N, 109°5'E	2	December 1995
Siberian whitefish or pidschian, <i>Coregonus lavaretus pidschian</i> Gmelin				
	Baikal basin:			
	Verkhyaya Angara River	55°70'N, 110°00'E	2	September 1998
	Bargusin River	53°65'N, 109°75'E	2	September 2000
	ªYenisey basin:			
	Irkut River	52°00'N, 103°75'E	1	August 1998
	ªUpper Lena River basin:			
	Lake Leprindo	56°70'N, 117°50'E	2	July 1998
	Lake Nichatka	57°40'N, 117°85'E	2	July 1998
	Lake Oron	57°50'N, 116°25'E	2	August 2000
	Lake Kapilyushi	55°87'N, 112°30'E	2	August 2000
	Ichikta River	56°60'N, 107°50'E	2	June 1999
Arctic cisco ( <i>Coregonus autumnalis</i> Pallas)				
	Laptev Sea:			
	Lena River delta	73°10'N, 129°40'E	2	April 1998
Irish pollan ( <i>Coregonus pollan</i> Thompson)				
	N. Ireland:			
	Lough Neagh	54°65'N, 6°35'W	2	October 1996

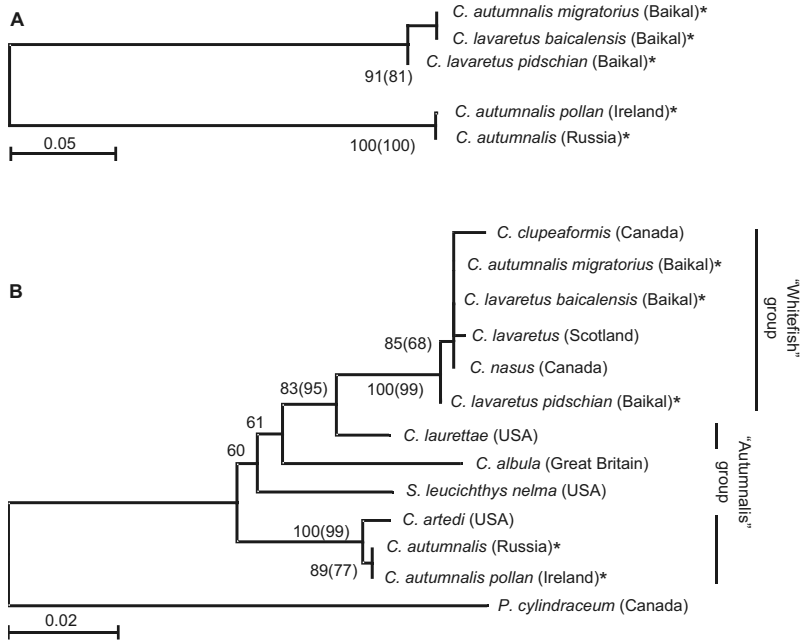
ª Additional individuals of *C. lavaretus pidschian*, which were screened for rDNA ITS1 length variation using PCR amplification.



**Fig. 1.** Scheme of variation in the coregonid ITS1 of rDNA based on sequence alignment of 13 taxa. Asterisks indicate the sequences of five taxa obtained by the authors (accession numbers in the EMBL Database are AJ417728 through AJ17732). Other sequences are from Sajdak and Phillips (1997). Numbers indicate sequence sites in the alignment. Dots (.) indicate invariant sites. Dashes (–) indicate a deletion or insertion event. 1st, 2nd and 3rd copies indicate palindrome sequences that include all sites (variable and invariant); in the rest, parts of aligned sequence invariant sites are excluded. Black square indicates phylogenetically unique informative mutations in *C. l. pidschian* sequence. “Whitefish” type sequences are framed. See text for details.

including fourteen Baikal omul (666 bp), two Baikal lacustrine whitefish (666 bp), and four specimens of Baikal pidschian (664 bp). No sequence variation was revealed within the taxa. The nucleotide sequence data that were obtained were deposited in the EMBL Nucleotide Sequence Database under accession numbers AJ417728 through AJ417732. The resulting nucleotide sequences were aligned together with the ITS1 sequences of other coregonids that had been published earlier (Sajdak & Phillips 1997), which gave a final data set of 748

bp. The comparison of the aligned sequences revealed 117 variable nucleotide positions (15.6% of all the sites assayed), and 32 parsimony informative sites (27.4% of the variable sites). The base composition was biased, but it was similar among all the taxa. Nucleotides G, A, T and C made up on average 31.8%, 14.7%, 19.5% and 34.0% of sequences. The average pair-wise transversion/transition ratio was 0.6. Figure 1 shows a scheme of variable sites in the coregonid rDNA ITS1 based on sequence alignment. Only the differences resulting from



**Fig. 2.** Neighbor-Joining trees inferred from nucleotide sequences of the first internal transcribed spacer of ribosomal DNA (rDNA ITS1). Numbers at the nodes outside of parentheses indicate NJ bootstrap values and MP bootstrap values are contained within parentheses. Taxa sequenced by authors are marked by asterisks: — **A:** tree constructed using sequences obtained by authors for five coregonid taxa; — **B:** tree constructed using sequences obtained by authors for five coregonid taxa combined with the other sequences of coregonids reported by Sajdak and Phillips (1997).

different numbers of adenines in the runs at the 3' end were revealed in the pair *C. pollan*/*C. autumnalis*. The ITS1 sequences of Baikal omul and Baikal lacustrine whitefish were identical. Baikal pidschian was distinguished from the rest of the whitefishes (including Baikal omul and Baikal lake whitefish) by two phylogenetically informative mutations (Fig. 1). These differences allowed identification of hybrid specimens among the littoral morphotypes of Baikal omul from the Barguzin River. Two specimens (from 10 sequenced) of Baikal omul (Barguzin River) had the same ITS1 sequences as all other omul that were studied, but their mtDNA was that of the Baikal pidschian (data not shown). The ITS1 length polymorphism revealed by Sajdak and Phillips (1997) that resulted from a different number of copies of a palindrome (65 bp in ciscoes, 66 or 67 bp in *C. lavaretus*) was confirmed: one copy was found in ciscoes and two copies in whitefishes. Baikal omul, as well as whitefishes, had two copies of the palindrome. All additional

samples (Table 1) of *C. lavaretus pidschian* had also two copies of the palindrome. All sequences of the second copy of the palindrome were identical.

When phylogenetic trees were constructed using the five sequences that had been obtained, both NJ and MP methods produced trees with two highly supported clades (Figs. 1 and 2a). One clade joined *C. autumnalis* and *C. pollan*, and another joined all Lake Baikal coregonids. The range of percent sequence divergence within the clades and between the clades was 0%–0.2% and 3.8%–4.0%, respectively. Such relationships persisted in other trees when the obtained sequences were combined with the other sequences of coregonids reported by Sajdak and Phillips (1997; Figs. 1 and 2b). In both NJ and MP trees, there were two highly supported monophyletic clades: one consisted of *C. artedi* (North America), *C. autumnalis* (Russia) and *C. pollan* (Ireland) and another combined all whitefishes together with their Lake Baikal representa-

tives. The range of percent sequence divergence within and between two highly supported clades also remained similar and was 0%–0.8% and 3.7%–5.0%, correspondingly. Because they were identical, Baikal omul, *C. autumnalis migratorius*, and Baikal lacustrine whitefish, *C. lavaretus baicalensis*, were retained within the whitefishes. Baikal pidschian, *C. lavaretus pidschian*, was basal to all other whitefishes because of two phylogenetically informative mutations.

## Discussion

The phylogenetic reconstructions based on rDNA ITS1 that were performed in this study were concordant with the analyses performed on the mtDNA sequence variation in the same five coregonid taxa (Fig. 2; Sukhanova et al. 2002). Firstly, both markers showed that Baikal omul, *C. autumnalis migratorius* Georgi, belongs to the *C. lavaretus* complex but not to the *C. autumnalis* group. Secondly, the complete ITS1 sequence similarity of Baikal lacustrine whitefish, *C. lavaretus baicalensis* Dyb. and Baikal omul confirmed close proximity of these fishes that was revealed by mtDNA analysis (Fig. 1). And thirdly, the basal location of Baikal pidschian, *C. lavaretus pidschian* Pallas, with respect to all whitefishes was well correlated with the high values for genetic distances between Baikal omul and Baikal pidschian that were obtained from the sequences of the control region (1.96%) and cytochrome *b* (1.18%) of mtDNA (Sukhanova 2002).

Thus, the use of nuclear DNA marker has once more demonstrated that the Baikal omul belongs to the *C. lavaretus* complex. Our results strongly suggest that Baikal lacustrine whitefish and Baikal omul (*C. autumnalis migratorius* Georgi) diverged from a common ancestor quite recently. The results of the investigations do not support the hypothesis of an hybrid origin for Baikal omul (Politov 2000, Sukhanova 2002). Among 14 analyzed specimens of Baikal omul, none had alleles that were characteristic of Arctic cisco. The potential hybrid origin for Baikal omul stems from its recognized ability to hybridize with Baikal whitefishes. At present, Baikal omul actively hybridizes with the geneti-

cally-remote Baikal pidschian (Skryabin 1969). In this study, we found hybrid specimens of Baikal omul bearing mtDNA of Baikal pidschian (data not shown). Natural hybridization between these fish takes place because pidschian and omul spawn in the same rivers, and occasionally the spawning times of pidschian and omul overlap. As for the closely related Baikal lacustrine whitefish, it is completely isolated from Baikal omul. Baikal omul migrates for spawning to the tributaries of Lake Baikal in September–October, and Baikal lacustrine whitefish spawns in the inner parts of bays in November–January (Skryabin 1969). Only artificial hybrids have ever been obtained for these forms (Mamontov 1988). Additionally, the results of population analyses of Baikal omul (Sukhanova et al. 1996) and Baikal lacustrine whitefish (Slobodyanyuk et al. 1993, Brzuzan 1998, Brzuzan et al. 1998) do not support a hypothesis of hybrid origin for Baikal omul. According to these studies, mtDNA diversity of Baikal omul is higher than that of Baikal lacustrine whitefish, but not vice versa, as would have been expected assuming omul to be a hybrid between Arctic cisco and the Baikal lacustrine whitefish.

Adding the five taxa that we studied to the ITS1 phylogenetic reconstruction of coregonid fish from Sajdak and Phillips (1997) resulted in two highly supported monophyletic clades (Fig. 2b). The first unites all whitefishes together with the Lake Baikal coregonids, and another consists of the ciscoes, *C. autumnalis* (Russia), *C. pollan* (Ireland) and *C. artedi* (North America). Hereinafter, we call these ciscoes the “autumnalis” group. The “whitefish” group includes all representatives of the *C. lavaretus* complex and the broad whitefish (*C. nasus*). The ITS1 length polymorphism from the added taxa, including the repeating units of the 65–67 bp palindrome, corresponds to that found in coregonid fishes (Fig. 1). Like other whitefishes, Baikal whitefishes and Baikal omul have the second repeating unit. Their first repeat has the same fixed differences at bases 110 and 123, which distinguishes the whitefishes from other coregonids. Their second repeat differs from the first and is identical in all sequenced taxa. This finding confirms the supposition of Sajdak and Phillips (1997) that the peculiarities of the second

repeating unit indicate that the duplication was a single event. The occurrence of the second repeat in only the whitefish group verifies the monophyly of that group. The ITS1 sequences of all representatives of the “autumnalis” group were similar (including the absence of the second copy of the palindrome). The Irish pollan is the closest to the Arctic cisco, and the results are congruent with previous molecular-phylogenetic analyses of coregonids. The representatives of the “autumnalis” group, as well as whitefishes, are monophyletic in the allozyme tree presented by Bodaly *et al.* (1991) and on the mtDNA tree obtained by restriction analysis of mtDNA presented by Bernatchez *et al.* (1991). There was a high degree of concordance between the trees. Later on, phylogenetic relationships among members of the Coregonidae were also inferred from sequencing the mtDNA fragments of Cyt *b* (Lockwood *et al.* 1993) and the d-loop (Reist *et al.* 1998). The bootstrap values of the Cyt *b* and d-loop trees obtained in both studies were insignificant due to the shortness of the sequenced mtDNA fragments. However, the topology of the trees agreed with that based on the allozymes (Bodaly *et al.* 1991) or the mtDNA restriction analyses mentioned above (Bernatchez *et al.* 1991). Similar relationships remained when three more whitefishes (Lake Baikal coregonids) and two representatives of the “autumnalis” group (*C. autumnalis* from Russia and *C. pollan* from Ireland) were added to Cyt *b* and d-loop phylogenetic reconstructions of the authors mentioned above (Sukhanova *et al.* 2002). The only disagreement of the ITS1 phylogenetic reconstruction with previous molecular-phylogenetic analyses of coregonids is the distinct position of the Bering cisco, *C. laurettae* (Fig. 2b). At first glance it would seem that *C. laurettae* drops out completely from the “autumnalis” group, while both allozyme and mtDNA markers testify to its affinity (Bodaly *et al.* 1991, Bernatchez *et al.* 1991, Lockwood *et al.* 1993, Reist *et al.* 1998). However, on closer visual examination of the alignment (Fig. 1) it is evident that although the second half of the *C. laurettae* sequence is almost identical to the sequence of *C. lavaretus*, its first half is identical to that of the “autumnalis” group (including the absence of the second copy of the palindrome). The accumulation of substitutions

is unlikely to have happened randomly. This “chimera” sequence is most likely the product of recombination of two different nuclear genomes (hybridization effect) or an artifact of the data handling processing (e.g. an error occurred as a result of computer processing of sequences). Thus, the ITS1 sequence analysis of five more coregonid taxa helped to confirm close relationships within two polymorphic groups of the Coregonidae: the *C. lavaretus* complex and the “autumnalis” group, which had been revealed earlier in allozyme and mtDNA surveys.

## Conclusion

The results of our study do not support the hypothesis of a hybrid origin for the Baikal omul. Undoubtedly, it will be necessary to carry out detailed population analyses of the three Lake Baikal coregonids to trace their origins. However, it is obvious that the systematic status of Baikal omul should be revised. We conclude that the Baikal omul is a member of the highly diverse *Coregonus lavaretus* complex. This complex combines numerous true whitefishes of North America (*C. clupeaformis*) and Eurasia (*C. lavaretus*) and is recognized as a single, variable and circumpolar ranging species, *C. lavaretus*, according to the most ancient name in use (Bernatchez & Dodson 1994). *Coregonus lavaretus* belongs to the group of true whitefishes with a lower mouth, although the mouth location can vary from typically low to almost terminal. It has many ecological forms distinguished from each other by a number of morphological features, places and terms of spawning. They are anadromous, riverine and lacustrine true whitefishes. In large lakes, they form coastal, deep-water and pelagic groups with different types of feeding — from typical planktophages to typical benthophages (Reshetnikov 2002). All the forms inhabit Lake Baikal. Baikal pidschian is a coastal benthophage, inhabits depths not lower than 30 m, spawns in rivers and stays there for a long time. Baikal lacustrine whitefish, is a deepwater benthophage, lives at depths up to 200 m, but mainly inhabits the depth of 30–100 m. It spawns in the lake (Skryabin 1968). Baikal omul has a terminal mouth, a large number of gill rakers,

morphological characteristics of fish that feed in the pelagic zone, and is one of the extreme variants of planktophage in the wide range of phenotypical diversity of the true whitefishes. Baikal omul inhabits the food rich pelagic zones of Lake Baikal up to depths of 350–400 m. These zones are: the epipelagic zone, benthic layers of the underwater slope, and the costal-pelagic area. Baikal omul enters the rivers only for spawning (Smirnov 1992). Its general morphological similarity with Arctic cisco is the result of a pelagic lifestyle in the deepwater lake.

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