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GENERAL BIOLOGY

Nucleotide Sequences of the Mitochondrial Cytochrome Oxidase Subunit I (COI) Gene of Lamprey Classified with *Lethenteron camtschaticum* and the *Lethenteron reissneri* Complex Show no Species-Level Differences

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Life history strategy has traditionally played an important role in the taxonomy of lampreys. In some cases, this was the basis for distinguishing so-called satellite species groups [1]. Lamprey larvae within a satellite species group are morphologically identical; however, adult lampreys differ from one another in a number of species (primarily, the body length). It is believed that lampreys of some species migrate to large bodies of water (rivers, lakes, seas, and oceans) after metamorphosis, become parasites of bony fishes and begin to reproduce within 0.5–3 years, whereas lampreys of other species remain in their native river, become mature within 6–10 months, and then die [1–4]. As a rule, a satellite species group comprises one parasitic anadromous species and several satellite resident species (nonparasitic or parasitic), which are assumed to have sympatrically originated from the first species [1, 4].

In particular, it is generally believed that, in the Far East, there are the parasitic anadromous lamprey *Lethenteron camtschaticum* and the species *L. reissneri* that has sympatrically originated from it. This species was originally thought to have a common origin with *L. japonicum*; later, however, *L. japonicum* was established to be synonymous to the species *L. camtschaticum*, which was described earlier [5]. In addition, some authors report that the Siberian nonparasitic lamprey *L. kessleri,* which is currently classified with the same group as *L. camtschaticum* and *L. reissneri*, also occur in the Far East [6].

In recent years, Japanese researchers pay much attention to the genetic characteristics of Far Eastern nonparasitic lampreys [7, 8]. Studies on both nuclear and mitochondrial markers have shown that, in addition to *L. camtschaticum*, two unique forms of lampreys live in Japan and South Korea, *L.* sp. *N* and *L.* sp. *S*, each of which can be classified as a separate species on the basis of substantial genetic differences from all lampreys studied thus far [7]. At the same time, *L. kessleri* and *L. reissneri*, which have been described as separate species, have exhibited such a high genetic similarity with respect to both nuclear and mitochondrial genomes that it has been suggested that these species should be combined into a single *L. reissneri* complex [8].

The genetic differences between the resident nonparasitic lampreys of the *L. reissneri* complex and the anadromous parasitic *L. camtschaticum* remain an open question. However, this issue is of special importance because some researchers deny that the lamprey life-strategy characteristics are hereditary [3, 4].

At present, the genetic characteristic of a species is routinely given on the basis of the nucleotide sequence of the cytochrome oxidase subunit I (COI) gene in the mitochondrial genome (barcoding). It has been demonstrated that differences between most species of chordates, even closely related ones, are greater than 2% [9]. The subject of this study was the nucleotide sequence of a 1072-bp mtDNA fragment containing part of the COI gene sequence in resident and anadromous lampreys from the same river system, namely the system of the Utkholok River in the western Kamchatka Peninsula.

MATERIALS AND METHODS

Lampreys were caught during spawning in the years 2005 and 2006 (May and June) in the spawning grounds by means of electric fishing in such a way that spawning individuals of different forms were simultaneously included into analysis. Figure 1 shows the location of the catching sites. Table 1 shows the species of each individual lamprey identified on the basis of a set of morphological characters that have taxonomic significance [3], as well as the geographic site and year where and when it was caught.

In addition to 20 spawners and one smolt (a lamprey that had transformed and was migrating to the sea

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Fig. 1. Sites where lampreys were caught: (*1*) the Utkholok River spawning ground; (*2*) the Kalkaveem River spawning ground; (*3*) the site where a smolt was caught; (*4*) the Mysmont Brook where larvae were caught. The arrow shows the position of the river system in the Kamchatka Peninsula.

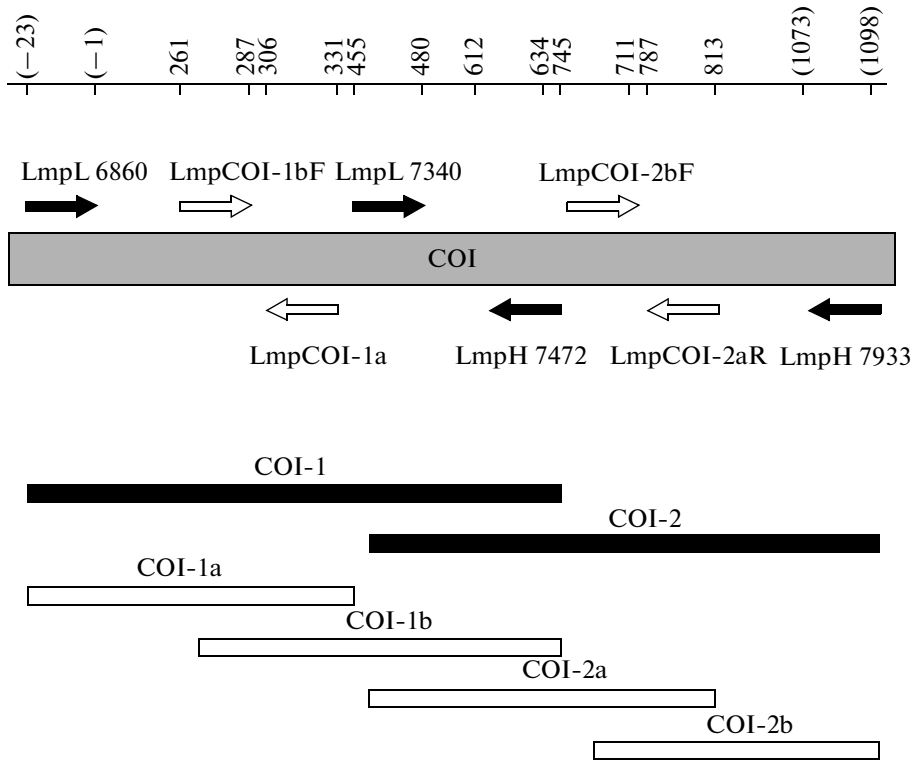

Fig. 2. The schematic diagram of the annealing of the main (black arrows) and additional (white arrows) primers within the mtDNA COI locus. At the top: a conventional (nonproportional) scale where the positions of the first and last nucleotides of each primer in the sequence deciphered in this study are indicated. At the bottom: PCR products obtained using different pairs of primers (black, only the main ones; white, both the main and additional ones).

for fatting), we analyzed five amocoete larvae that certainly belonged to the resident form, because anadromous parasitic lampreys have never been found in the shallow tributary where the larvae were caught (the Mysmont Brook), and, in turn, amocoete larvae never migrate upstream over considerable distances.

Samples of tissues (usually, a fragment of a fin) for genetic analysis were fixed with 96% ethanol (1 : 5).

DNA was isolated using a Diatom™ DNA Prep 200 reagent kit (Laboratoriya Izogen, Moscow, Russia) as recommended by the manufacturer.

In most cases, an mtDNA fragment for analysis was obtained in the form of two overlapping PCR products designated here as COI-1 (657 bp) and COI-2 (644 bp). For obtaining these PCR products, we used the pairs of primers (1) LmpL6860 and LmpH7472 and (2) LmpL7340 and LmpH7933, respectively [7]. For the cases where the DNA of the analyzed samples was too degraded for obtaining sufficiently long PCR products, we developed additional internal primers. Their use in combination with the primers listed above allowed us to obtain the truncated overlapping PCR products (1) COI-1a (357 bp) and COI-1b (374 bp) and (2) COI-2a (359 bp) and COI-2b (354 bp), respectively. Figure 2 schematically shows the positions of the primers in the mtDNA fragment analyzed in this study. The nucleotide sequences of all primers are shown in Table 2.

All PCR products were obtained by means of a Tertsik amplifier (DNK-Tekhnologiya, Moscow, Russia). Amplification was performed in 25 μl of a buffer solution (Fermentas) (75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.1% Tween 20, and 2 mM $MgCl_2$). The amplification mixture contained about 300 ng of total cellular DNA, 200 nmol of each of the four deoxyribonucleotides, 10 pmol of each of two (forward and reverse) primers, and 0.5–0.7 U of *Taq* polymerase (Bionem, Moscow, Russia). To prevent evaporation during the reaction, mineral oil was layered onto the mixture. The amplification procedure consisted of the initial denaturation stage (4 min at 95°C), 30 cycles of synthesis of the PCR product (45 s at 95°C, 45 s at 54°C, and 1 min at 72°C), and the final elongation stage (5 min at 72°C).

The resultant PCR products were reprecipitated at room temperature by adding, to the amplification mixture, ethanol to a final concentration of 70% and ammonium acetate to a final concentration of 125 mM. The precipitated DNA was washed with 70% ethanol, dried, and solved in bidistilled water.

About 0.3 pmol of the PCR product and 3.2 pmol of the corresponding primer were used in the sequencing reaction. Each PCR product was sequenced using both the forward and the reverse primers. DNA sequencing was performed with the use of the ABI PRISM® BigDye™ Terminator v. 3.1 reagent kit, by means of an ABI PRISM 3730 automated sequencer (Applied Biosystems) at the GENOM Collective Access Center, Engelhardt Institute of Molecular Biology, Russian Academy of Sciences (Moscow, Russia).

Table 1. Characteristics of the samples used for studying the mtDNA COI nucleotide sequence in Lampreys from the Utkholok River basin

Site of collection of the material	Species identified on the basis of a set of morphological and ecological characters	Year of collection of the material
Utkholok River	*L. camtschaticum*	2005
Utkholok River	*L. camtschaticum* (smolt)	2005
Utkholok River	*L. camtschaticum*	2005
Utkholok River	"	2005
Utkholok River	*L. reissneri* complex	2005
Kalkaveem River	*L. camtschaticum*	2005
Kalkaveem River	"	2005
Kalkaveem River	"	2005
Kalkaveem River	"	2005
Kalkaveem River	"	2005
Kalkaveem River	*L. reissneri* complex	2005
Kalkaveem River	"	2005
Kalkaveem River	"	2005
Kalkaveem River	"	2005
Kalkaveem River	"	2005
Kalkaveem River	"	2005
Kalkaveem River	"	2005
Kalkaveem River	"	2005
Kalkaveem River	"	2005
Kalkaveem River	"	2005
Kalkaveem River	"	2006
Mysmont Brook	*L.* sp. (ammocoet) = (*L. reissneri* complex)	2006
Mysmont Brook	"	2006
Mysmont Brook	"	2006
Mysmont Brook	"	2006
Mysmont Brook	"	2006

The results of sequencing were analyzed using the BioEdit v. 7.0.5 software [10].

RESULTS AND DISCUSSION

All the sequenced PCR products are presented in the GenBank international database. The accession numbers and specific characteristics of the sequences are shown in Table 3.

The full-length sequence of the mtDNA COI locus was 1072 bp in size for all sequenced samples, the sequences differing from one another by no more than three nucleotide substitutions (i.e., by less than 0.3%) in each pairwise comparison. In total, five variable nucleotides were found in the sequenced fragment, which corresponded to less than 0.5% of the nucle-

Table 2. The primers used for obtaining PCR products and sequencing of the mtDNA COI locus in lampreys

Primer	Nucleotide sequence	Source
LmpL 6860	5'-GGCTTTGGCAACTGACTTGTACC-3'	[7]
LmpCOI-1aR	5'-GAGGACTGCAGTAATTAAAACGGATC-3'	this study
LmpCOI-1bF	5'-TAAARCCYCCAACTATAACACAATACC-3'	this study
LmpH 7472	5'-TACTGTGAATATGTGRTGGGCTC-3'	[7]
LmpL 7340	5'-TGATTTTTTGGTCACCCTGAAGTTTA-3'	[7]
LmpCOI-2aR	5'-CCGGTRAGTCCYCCTACAGTRAATAAG-3'	this study
LmpCOI-2bF	5'-ATGACACACCCCCATACTATGRGC-3'	this study
LmpH 7933	5'-CATGTAGTGTATGCATCAGGGTARTC-3'	[7]

Table 3. The positions of variable nucleotides in the nucleotide sequence of the mtDNA COI locus in lampreys from the Utkholok River basin

Species identified on the basis of a set of morphological and ecological characters	Positions of variable nucleotides					Accession no. of the sequence in the GenBank
	304	451	775	1036	1057	
L. camtschaticum	A	G	A	T	A	HQ686281
L. camtschaticum (smolt)	T	A	A	T	G	HQ686282
L. camtschaticum	T	G	A	T	A	HQ686283
L. camtschaticum	A	G	A	T	A	HQ686284
L. reissneri complex	T	G	A	T	A	HQ686285
L. camtschaticum	T	G	A	T	G	HQ686286
L. camtschaticum	T	G	A	T	A	HQ686287
L. camtschaticum	T	G	A	T	G	HQ686288
L. camtschaticum	T	G	A	T	G	HQ686289
L. camtschaticum	T	G	A	T	G	HQ686290
L. reissneri complex	T	G	A	T	A	HQ686291
L. reissneri complex	T	G	A	T	A	HQ686292
L. reissneri complex	T	G	A	T	A	HQ686293
L. reissneri complex	T	G	A	T	A	HQ686294
L. reissneri complex	A	G	A	T	A	HQ686295
L. reissneri complex	A	G	A	T	A	HQ686296
L. reissneri complex	T	A	A	T	G	HQ686297
L. reissneri complex	T	G	A	T	G	HQ686298
L. reissneri complex	T	G	A	C	G	HQ686299
L. reissneri complex	T	G	A	T	A	HQ686300
L. reissneri complex	A	G	A	T	A	HQ686301
L. sp. (ammocoet)=(*L. reissneri* complex)	T	G	A	T	A	HQ686302
L. sp. (ammocoet)=(*L. reissneri* complex)	A	G	A	T	A	HQ686303
L. sp. (ammocoet)=(*L. reissneri* complex)	T	G	A	T	A	HQ686304
L. sp. (ammocoet)=(*L. reissneri* complex)	T	G	A	T	A	HQ686305
L. sp. (ammocoet)=(*L. reissneri* complex)	T	G	G	T	G	HQ686306

otide sequence. Figure 3 shows the sequence that was the most frequent in lampreys from the Utkholok River basin (in 11 out of 26 cases); the nucleotide positions where substitutions were found are framed.

We found four different mtDNA COI haplotypes in the group of nine anadromous specimens, five haplotypes in the group of 12 resident spawners, and three haplotypes in five amocoete larvae classified with the *L. reissneri* complex.

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5'-  TATAATACTT  AGCGCCCCTG  ATATAGCCTT  CCCACGTATA  AATAACATAA
  51 GCTTTTGACT  GCTCCCACCA  TCCCTACTCT  TACTTTTAGC  TTCCGCAGGA
 101 GTTGAAGCAG  GAGCCGGAAC  TGGATGAACA  GTATACCCAC  CTCTAGCAGG
 151 AAATTTAGCC  CACACAGGGG  CCTCTGTTGA  CTTAACAATT  TTCTCCCTTC
 201 ATCTAGCCGG  TATTTCATCA  ATCCTTGGGG  CAGTCAACTT  TATTACAACA
 251 ATTTTTAACA  TAAAACCTCC  AACTATAACA  CAATACCAAA  CCCCATTATT
 301 TGT[T]TGATCC  GTTTTAATTA  CTGCAGTCCT  CCTTCTTCTA  TCACTTCCTG
 351 TACTTGCAGC  TGCCATCACT  ATACTTTTAA  CAGATCGTAA  TTTAAATACA
 401 TCCTTCTTTG  ACCCTGCAGG  AGGAGGAGAC  CCAATCCTTT  ACCAACACCT
 451 [G]TTCTGATTC  TTTGGGCACC  CTGAAGTTTA  TATTCTAATT  TTACCAGGCT
 501 TTGGAATTAT  CTCTCACGTA  GTCGCCTACT  ACTCCGGGAA  AAAAGAACCA
 551 TTTGGATATA  TAGGAATAGT  CTGAGCAATA  ATAGCCATTG  GGTTACTAGG
 601 GTTTATTGTA  TGAGCCCACC  ACATATTCAC  GGTAGGAATA  GATGTTGACA
 651 CACGAGCCTA  CTTTACATCA  GCCACAATAA  TTATTGCTAT  TCCAACAGGA
 701 GTTAAAGTCT  TTAGCTGACT  AGCCACACTC  CATGGCGGAA  AAATCGTGTG
 751 ACACACCCCC  ATACTATGAG  CCCT[A]GGCTT  CATTTTCTTA  TTTACTGTAG
 801 GGGGACTTAC  CGGAATTGTC  TTATCCAACT  CATCACTAGA  TATTATCCTT
 851 CATGATACTT  ATTATGTAGT  AGCTCACTTC  CATTATGTAT  TATCTATAGG
 901 AGCTGTTTTC  GCAATCATAG  CTGGCTTCGT  TCATTGATTC  CCACTATTTA
 951 CAGGATATAC  ACTTAACGAA  ACCTGATCAA  AAGCACACTT  TGTCATTATA
1001 TTTACTGGTG  TAAATCTTAC  ATTCTTCCCT  CAACA[T]TTCC  TAGGTTTAGC
1051 TGGTAT[A]CCA  CGACGTTACT  CA -3'
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Fig. 3. The variant of the nucleotide sequence of the mtDNA COI locus that is the most common in lampreys from the Utkholok River basin. The nucleotides that vary in different individuals of this aquatic system are framed.

All haplotypes identified in anadromous lampreys were also found in resident spawners. One haplotype found in a resident spawner was absent in all other groups, and so was one of the haplotypes found in a resident amocoete larva. Two haplotypes found in this last group were common for all groups studied. Table 3 shows all nucleotide substitutions in the mtDNA COI locus of individual lampreys that we found in this study.

Thus, the analyzed fragment of mtDNA proved to contain no nucleotides specific for either resident or anadromous lampreys of the Utkholok River; i.e., there were no species-level differences between resident and anadromous lampreys from the Utkholok River basin. Note that the number of identified haplotypes also depended on the sample size rather than the life history form of the studied group of lampreys. Indeed, along with three most common haplotypes, whose frequencies were 42, 23, and 19%, there were three rare haplotypes found in 4–8% lampreys from the Utkholok River (i.e., in one or two individuals each). Given these data, the observed diversity of mtDNA COI variants within individual groups of lampreys from the Utkholok River was almost the same in the resident and anadromous forms, the proportion of variable nucleotides being low.

Note that the data suggesting that the species *L. camtschaticum* and the *L. reissneri* complex are not genetically separate entities do not contradict the results earlier reported by other authors [8]. For example, the same mtDNA COI haplotype was found in an individual from the Byeraya River (a tributary of the Naiba River, Sakhalin) classified with *L. kessleri* and two individuals from the Japanese Islands classified with *L. camtschaticum* (*L. japonicum* in the original paper). Moreover, this haplotype is very likely to be the same mtDNA COI haplotype that is the most frequent in lampreys from the Utkholok River basin. Anyway, the common portion of the 1009-bp sequences for the above individuals presented in the GenBank (AB198751, AB198746, and AB198747) and the sequence reported here (Fig. 3) is absolutely the same in all these cases (the mtDNA fragment sequenced in our study contains 26 additional nucleotides at the 5' end and 37 additional nucleotides at the 3' end, some of them being variable).

It is also noteworthy that, in all phylogenetic trees reported to date [4, 8, 11], the differences between the lampreys classified with *L. camtschaticum* and the *L. reissneri* complex are nonsignificant, although the authors compare the nucleotide sequences of the COI and *cyt b* mtDNA loci of representatives of *L. camtschaticum, L. kessleri,* and *L. reissneri* from geographically remote places.

The results of our study confirm the hypothesis that the resident and anadromous lampreys from the Utkholok River basin belong to the same species (*L. camtschaticum*). This hypothesis was suggested earlier on the basis of overlapping of the morphological characters regarded as species-specific in different

forms of lampreys [3], as well as observations of joint spawning of the resident and anadromous lampreys [2]. Thus, our data are evidence in favor of the assumption that most characteristics of life history strategy in lampreys are nonhereditary and are mainly determined by environmental factors.

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REFERENCES

1. Hardisty, M.W., *Life without Jaws*, Ceredigion: Forrest, 2006.
2. Kucheryavyi, A.V., Savvaitova, K.A., Gruzdeva, M.A., and Pavlov, D.S., *Vopr. Ikhtiol.*, 2007, vol. 47, no. 4, pp. 462–466.
3. Kucheryavyi, A.V., Savvaitova, K.A., Pavlov, D.S., et al., *Vopr. Ikhtiol.*, 2007, vol. 47, no. 1, pp. 42–57.
4. Docker, M.F., *Am. Fish. Soc. Symp.*, 2009, vol. 72, pp. 71–114.
5. Bogutskaya, N.G. and Naseka, A.M., *Katalog beschelyustnykh i ryb presnykh i solonovatykh vod Rossii s nomenklaturnymi i taksonomicheskimi kommentariyami* (The Catalogue of Agnathans and Fishes of Fresh and Brackish Waters of Russia, with Nomenclatural and Taxonomic Comment), Moscow: KMK, 2004.
6. *Atlas presnovodnykh ryb Rossii* (The Atlas of Freshwater Fishes of Russia), Reshetnikov, Yu.S., Ed., Moscow: Nauka, 2002, vol. 1.
7. Yamazaki, Y., Goto, A., and Nishida, M., *J. Fish Biol.,* 2003, vol. 62, pp. 591–609.
8. Yamazaki, Y., Yokoyama, R., Nishida, M., and Goto, A., *J. Fish. Biol.*, 2006, vol. 68, suppl. B, pp. 251–269.
9. Hebert, P.D.H., Ratnasingham, S., and de Waard, J.R., *Proc. R. Soc. Lond. B,* 2003, vol. 270, suppl., pp. S96–S99.
10. Hall, T. and Therapeutics, I., *Biological Sequence Alignment Editor for Win 95/98/NT/2K/XP, BioEdit vers. 7.0.5* (http://www.mbio.ncsu.edu/BioEdit/bioedit.html)/
11. Lang, N.J., Roe, K.J., Renaud, C.B., et al., *Am. Fish. Soc. Symp.*, 2009, vol. 72, pp. 41–55.