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## TAXONOMIC IMPLICATIONS FOR THE RIPUS, *COREGONUS ALBULA* INFRASP. *LADOGENSIS* FROM MITOCHONDRIAL DNA ANALYSIS

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**ABSTRACT.** The nucleotide sequence (223 bp) of the part of mitochondrial DNA (mtDNA) control region (CR) was determined in a formalin-preserved specimen of ripus, *Coregonus albula* infrasp. *ladogensis*. This fish, a representative of introduced coregonid stock that is now extinct in Poland, was caught in 1962 in Lake Radomno in northeastern Poland. The obtained CR sequence was compared to that of other coregonid species, such as European whitefish (*C. lavaretus*), vendace (*C. albula*), peled (*C. peled*) as well as North American lake cisco (*C. artedii*) and Arctic cisco (*C. autumnalis*). The results revealed that the ripus sequence was highly similar to that of both cisco species. Phylogenetic analysis with Bayesian inference placed the individual ripus sequence into the ciscoes' clade with a high clade credibility value  $f(\tau | CR) = 0.86$ . The results improve the knowledge of the taxonomic position of *Coregonus albula* infrasp. *ladogensis*, which occurs naturally in Lake Ladoga.

**Key words:** RIPUS (*COREGONUS ALBULA* INFRASP. *LADOGENSIS*), FORMALIN-PRESERVED SPECIMEN, mtDNA SEQUENCING, TAXONOMY

## INRODUCTION

Ripus, *Coregonus albula* infrasp. *ladogensis* (Berg 1948), is a deep-water coregonid fish that occurs naturally in Lake Ladoga (Russia) where it lives in sympatry with vendace (*Coregonus albula* (L.)). Due to the scarcity of available data, the taxonomical status of the ripus from Lake Ladoga remains unclear (Kottelat 1997). The possibility of sequencing DNA from formalin-preserved museum specimens provides scientists with a promising sampling strategy for phylogenetic studies of fish (Brzuzan and Ciesielski 2000).

In the late 1950s Poland received ripus eggs from Russian hatcheries (Bernatowicz 1964). However, the introduction failed and the species disappeared from Polish lakes in the late 1960s. Lake Radomno in northeastern Poland was one of the few lakes where both stocking with ripus and commercial catches of it were documented (A. Kapusta, Inland Fisheries Institute in Olsztyn, personal communication).

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TABLE 1

Nominate taxa of the genera *Coregonus* and *Prosopium* used in the study, with location sites of populations that provided samples. In this study the mtDNA sequence was obtained from taxon No. 1, whereas the sequences from taxa No. 2 through 7 were determined previously by Reist et al. (1998)

No.	Taxon	Population
1.	Ripus ( <i>Coregonus albula</i> <i>infrasp. ladogensis</i> )	Lake Radomno, Poland
2.	Vendace ( <i>Coregonus albula</i> (L.))	Lake Inari, Finland
3.	Peled ( <i>Coregonus peled</i> (Gmelin))	Middle River Ob, Russia
4.	Whitefish ( <i>Coregonus lavaretus</i> (L.))	Lake Inari, Finland
5.	Lake cisco ( <i>Coregonus artedii</i> Lesueur)	Churchill River, Maintoba, Canada
6.	Arctic cisco ( <i>Coregonus autumnalis</i> (Pallas))	Arctic Red River, Northwest Territories, Canada
7.	Round whitefish ( <i>Prosopium cylindraceum</i> (Pennant))	Chatanika River, Alaska, USA

In order to improve knowledge regarding the taxonomic position of ripus, the authors determined the nucleotide sequence of the part of mitochondrial DNA (mtDNA) control region (CR) in a formalin-preserved specimen of a fish from a coregonid stock that was introduced but is now extinct in Poland. Based on the CR sequences published in the literature (Reist et al. 1998), the authors inferred the genetic relationships between the ripus and other taxa within the subfamily Coregoninae.

## MATERIAL AND METHODS

The fish used in this study was caught in Lake Radomno in 1962, then it was preserved in a 4% formaldehyde solution and stored at the Inland Fisheries Institute in Olsztyn, Poland.

The DNA extraction method used in the study was described previously by Brzuzan and Ciesielski (2000). Small pieces of gill (each about 0.4 g of tissue) were removed from the specimen and washed in 20 volumes of TE9 buffer (500 mM Tris, 20 mM EDTA, 10 mM NaCl, pH 9.0) for 24 hours. The samples were then placed into 1.5 ml centrifuge tubes containing 1 ml of digestion solution (TE9 buffer, 4 mg Proteinase K, and 10 mg SDS), and incubated at 50°C for 72 hours. After 24 and 48 hours, an additional 4 mg of Proteinase K and 10 mg SDS was added to each tube. During the incubation process the sample was vortexed several times a day. The digested tissue was extracted twice with phenol (0.5 ml) and once with chloroform – isoamyl alcohol (24:1). The aqueous phase was transferred into another 1.5 ml tube containing 0.1 volume of

3M sodium acetate solution. The DNA was precipitated with 0.6 ml of isopropanol for 15 min and then the samples were centrifuged at 12000 g for 15 min to obtain a DNA pellet. The pellet was washed with 70% ethanol, centrifuged again at 12000 g for 15 min, and air dried. Finally, the DNA pellet was resuspended in TE buffer (pH 8.0).

The DNA segment, spanning a part of the *tRNA<sup>Pro</sup>* gene and the right portion of mtDNA control region (CR), was amplified with a polymerase chain reaction primer set – L (5'-CCACTAGCTCCCAAAGCTA-3') and H2 (5'-CGTTGGTCGGTTCTTAC-3'), according to the protocol by Brzuzan (1998). Before sequencing, the PCR product was purified from oligonucleotides, primers, and dimers using GenElute PCR Purification Kit (Sigma). Sequencing was performed using a Perkin Elmer ABI 373 automated DNA sequencer, and the DyeDeoxy Cycling Sequencing reaction (PE-Applied Biosystems, California, USA) was performed at the Institute of Biochemistry and Biophysics in Warsaw, Poland. Approximately 250 base pairs from the mtDNA control region were determined for both strands. The ripus mtDNA sequence is deposited in GenBank under accession number AY438265. In this study a sequence of a 223 bp portion of the CR portion was aligned (ClustalV; Higgins et al. 1991) and compared with mtDNA sequences previously determined by Reist et al. (1998) for European whitefish, vendace, peled as well as for North American lake cisco and Arctic cisco (Table 1). The taxa examined represent three defined supra-specific groups within the subfamily Coregoninae (i.e. whitefishes, vendace-peleds, and ciscoes; Reist et al. 1998).

The phylogenetic relationships among the mtDNA sequences were inferred with Bayesian analysis using the Markov chain Monte Carlo (MCMC) method (Huelsenbeck et al. 2000, Huelsenbeck and Bollback 2001). The relationships were examined by approximating the posterior probabilities of the trees ( $\tau$ ) using the MrBAYES program (Huelsenbeck and Ronquist 2001). In the MCMC settings all the trees were *a priori* equally probable. The trees were rooted using round whitefish as the outgroup (Reist et al. 1998). The HKY85 (Hasegawa et al. 1985) model of DNA substitution was assumed with  $\Gamma$  rate variation (Yang 1994). This approach has recently proven successful for inferring the taxonomic position of a coregonid fish whose bone remains were found in lacustrine deposits of Pleistocene (Brzuzan et al. 2004). In this study, four Markov chains were run simultaneously for 50 000 generations, sampling the chains every 10 generations. The states of the chains that were sampled before generation 4 000 were discarded.

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C. a. i. ladogenesis AGAACGGTTCCGTTGGTGATTATCCGAGTCTTGCTTAATGTAAACCTTGGAACAGTACTG 60
C. artedi .....
C. autumnalis .....
C. albula .....G...
C. peled .....G...
C. lavaretus .....G.....

C. a. i. ladogenesis ATGTATGAGGGCTTAAAATCACTTATGTTGATAATACATAGGATGTACTACCCACGTGCA 120
C. artedi .....
C. autumnalis .....
C. albula .....C.....
C. peled .....C.....
C. lavaretus .....T.....

C. a. i. ladogenesis ATAATAATATATGGGTCAGTACATAATATGCAATATTATACATAGTGGTGAATATACAC 180
C. artedi .....
C. autumnalis .....
C. albula .....
C. peled .....
C. lavaretus .....

C. a. i. ladogenesis ACACTGTATGGGGGTATATAATTTACCATGTACATTGCGGCGC 223
C. artedi .....
C. autumnalis .....A....
C. albula .....A...G..C.....T
C. peled .....A...G..C.....T
C. lavaretus .....A...G.....C.....T

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Fig. 1. Ripus (*Coregonus albula* infraspp. *ladogenesis*) nucleotide sequence (223 bp) of the part of mitochondrial DNA control region (CR) aligned with the sequenced CR fragments from five coregonid species: *C. artedi*, *C. autumnalis*, *C. albula*, *C. peled*, and *C. lavaretus*. Identical positions are indicated by dots (.). The ripus CR sequence is deposited in GenBank under the accession number AY438265.

## RESULTS AND DISCUSSION

Ten variable nucleotide positions were found within a 223bp fragment of the CR analyzed in the study (Fig. 1). The ripus sequence matched the sequence of *C. artedi*, and differed from that of *C. autumnalis* by one substitution of G for A at position 219 (noted as G219A). Furthermore, the comparison of the obtained sequence with published CR sequences for European whitefish, vendace, and peled revealed, in all cases, six nucleotide differences (A54G; C113T; G194A; A200G; T215C; C223T), (A57G; T79C; G194A; A200G; T203C; C223T), and (A57G; T79C; G194A; A200G; T203C; C223T), respectively (Fig. 2).

The focus of the study was to determine the taxonomical position of the ripus. Therefore, Bayesian analysis of the mtDNA control region was performed in order to

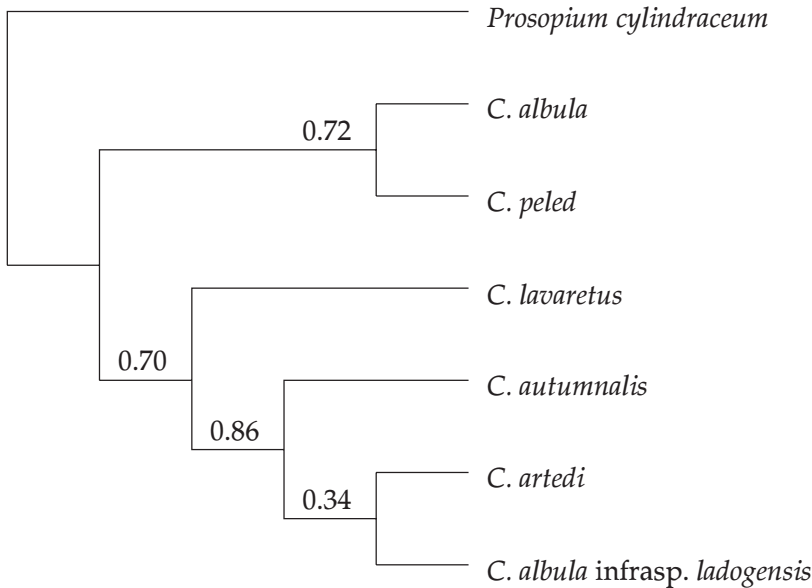


Fig. 2. Tree with the maximum posterior probability for the analyses of the mtDNA control region (CR) sequences of the ripus and other published CR sequences for European whitefish (*C. lavaretus*), vendace (*C. albula*), peled (*C. peled*), lake cisco (*C. artedi*) and Arctic cisco (*C. autumnnalis*). The numbers at the interior nodes represent the posterior probability that the clade is correct. The posterior probabilities of clades were approximated with the program MrBAYES (Huelsenbeck and Ronquist 2001). The tree was rooted using round whitefish (*Prosopium cylindraceum*) as the outgroup.

find its most probable phylogeny. Fig. 2 summarizes the results of the analysis. In general, the tree agreed with the coregonid phylogeny proposed by Reist et al. (1998) in that ciscoes grouped as sister taxa [posterior probability  $f(\tau | CR) = 0.86$ ], vendace and peled associated with each other as a consistent subgroup [ $f(\tau | CR) = 0.72$ ], and that whitefish associated with ciscoes [ $f(\tau | CR) = 0.70$ , Fig. 2]. The authors' belief regarding the phylogeny of the seven coregonine taxa was that in most reconstructions the ripus sequence would fall into one clade with *C. albula*. Interestingly, the tree with the largest posterior probability was that which included the ripus in one clade with ciscoes, i.e. (*P. cylindraceum*, (*C. albula*, *C. peled*), (*C. lavaretus*, (*C. autumnnalis*, (*C. artedi*, ripus))))); the posterior probability of this tree was  $f(\tau | CR) = 0.86$ . Since the posterior probabilities of the reconstructions that clustered the ripus with *C. albula*-*C. peled* pair were only  $f(\tau | CR) = 0.06$  (data not shown), on average, it is four-

teen times more likely that ripus represents the evolutionary (mitochondrial DNA) line of ciscoes (*C. artedi*-*C. autumnalis*) and is distinct from that of vendace (Fig. 2). The close genetic similarity of the ripus and the North American ciscoes may suggest that these taxa are racial forms of one species. Alternatively, the ripus and ciscoes represent separate species although they are not divergent at a level observed in other species. This view is consistent with other molecular studies of ciscoes (e.g., Reed et al. 1998) that show that in these fishes apparent phenotypic diversity (distinct morphology, pigmentation, gill-raker counts) may have occurred with only little genetic differentiation.

The current study questions the validity of considering the ripus as a subspecies of *Coregonus albula* as has been suggested by Berg (1948). However, the authors are aware that this study should be followed by other investigations that employ other genetic markers or morphological and ecological investigation, before the new taxonomic position (and nomenclature) of ripus becomes clear.

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## STRESZCZENIE

### ANALIZA MITOCHONDRIALNEGO DNA RIPUSA ŁADOSKIEGO, *COREGONUS ALBULA* INFRASP. *LADOGENSIS* – IMPLIKACJE TAKSONOMICZNE

W latach 60. ubiegłego wieku do niektórych jezior północnej Polski próbowano bez sukcesu wprowadzić ripusa ładoskiego (*Coregonus albula* infrasp. *ladogensis*). Z tamtego okresu pochodzi jedynie kilka muzealnych eksponatów tych ryb zakonserwowanych w formalinie. Porównanie sekwencji nukleotydowej fragmentu regionu regulacyjnego mtDNA ripusa (223 pary zasad) z jeziora Radomno, odłowionego w 1962 roku, z sekwencjami innych europejskich i północnoamerykańskich gatunków Coregoninae (tabela 1) udowodniło, iż ryba ta jest genetycznie znacznie bliższa północnoamerykańskiemu cisco (*C. artedii* i *C. autumnalis*) niż rodzimym sielawom (*C. albula*) (rys. 1 i 2). Ponieważ obowiązująca nazwa *Coregonus albula* infrasp. *ladogensis* traktuje ripusa jako podgatunek sielawy, uzyskany w tej pracy wynik może mieć implikacje taksonomiczne.