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Identification of whitefish remains by mtDNA analysis

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Remains of *Coregonus* sp. were obtained from the collection of archeological materials found in 1991 at the site Wola Grzymalina, Poland. DNA has been extracted from vertebrae that were dated about 500 000 years old. Polymerase chain reactions on extracts from the vertebral remains were succesful only using primers that targeted nucleotide sequences that were not longer than about 100bp. The ancient nucleotide sequence of a 102bp fragment of mitochondrial DNA *ND1* gene matched the contemporary sequence of European whitefish (*Coregonus lavaretus*) and differed from that of vendace (*C. albula*) by four substitutions. Ambiguous nucleotides were observed at nine positions throughout the 87bp fragment of mtDNA control region (*CR*), nonetheless the sequence was different from that of vendace by eight additional changes. Phylogenetic analysis utilizing Bayesian inference placed the archival sequence into a clade with European whitefish. The support for this reconstruction was $f(\tau|X) = 0.65$ after analysis of the *ND1* fragment and $f(\tau|X) = 0.52$ after analysis of the *CR* fragment. Excellent preservation of the specimen analysed in this study proves the potential for analysing genetic characteristics of species and specimens that are thousands of years old.

Introduction

Identification of preserved biological remains by DNA analysis is a powerful tool in forensic and historical studies (Hagelberg *et al*. 1991). Mitochondrial (mt) DNA is especially useful, because it is present at a high (up to several thousand) copy number in each cell, and at least some intact (i.e. not degraded) mtDNAs often persist in samples of ancient DNA (Hagelberg *et al*. 1991). It has been shown that mtDNA analysis can be performed successfully on human remains up to 12 000 years old (Hagelberg *et al*. 1989) and even on Neandertal skeletons (Krings *et al*. 1999, Ovchinnikov *et al*. 2000). The ancient DNA studies of mammal and bird material from field sites and museum collections have allowed inferences about prehistoric population sizes, phylogenetic relationships, mutation rates or pathways of disease transmission (Cooper & Wayne 1998, Lambert *et al*. 2002). Recently, Barnes *et al*. (2002) have used ancient DNA to reconstruct the migration patterns of subarctic brown bears living up to 60 000 years ago.

Archeological material from extinct fish taxa has been a rich source of DNA (e.g. Butler & Bowers 1998, Ciesielski *et al*. 2002). Genetic information retrieved from such samples may be particularly important when the systematic position of a taxon is inferred from morpho-

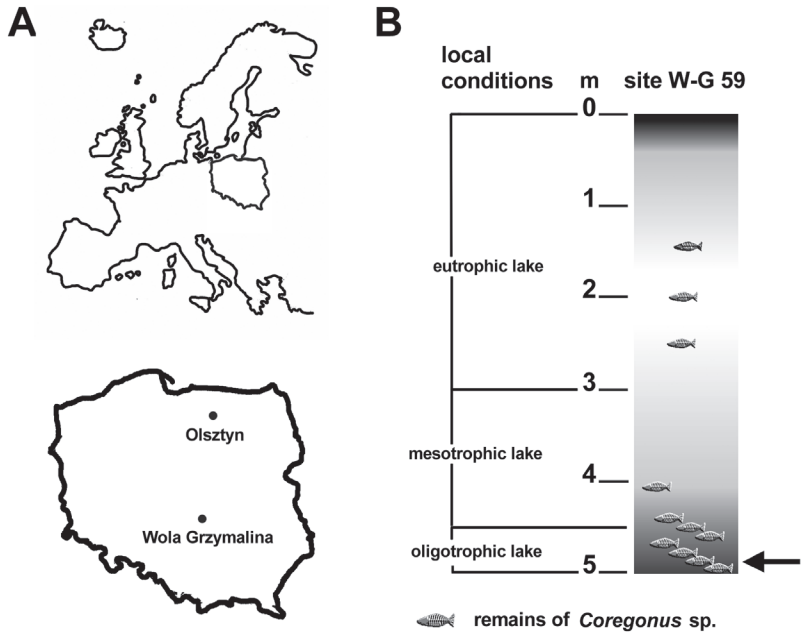

Fig. 1. (**A**) Location of Wola Grzymalina, and (**B**) positions of the *Coregonus* sp. remains in the section on site W-G 59 of the lacustrine deposits in the Ferdynandovian Interglacial. Most of the remains were noticed at a depth between the 4th and 5th meter of the section. The stratigraphic position of a specimen W-G 59/5 examined in this study is indicated by an arrow. Trophic phases (local conditions) of the lake after Krzyszkowski (1991). (Modified from Jerzmańska & Raczyński 1991).

logical characters. In this paper we describe how available methods for extracting and studying ancient mtDNA were used to identify archeological material as European whitefish, *Coregonus lavaretus* (Salmonidae, Teleostei).

In 1991, an assemblage of freshwater teleosts was described from lacustrine deposits of Pleistocene age in Poland, Wola Grzymalina (Jerzmańska & Raczyński 1991; Fig. 1). Palynological investigations ascribed the lacustrine deposits to the lower optimum phase of the Ferdynandovian Interglacial (Kuszell 1991). This Interglacial (younger than Elsterian, but older than Holsteinian) occurred some 500 000 years before present (Janczyk-Kopikowa *et al*. 1981). The pollen analyses (Kuszell 1991) have confirmed that the deposits of the lower part of the section from which the teleosts' remains come, had accumulated in a cool climate of the pre-optimum phase (taiga).

Incomplete skeletons and scales were among the best preserved materials found there, and about 80 isolated scales were identified as originating from *Coregonus*. To our knowledge, the finding represents the first occurrence of *Coregonus* remains in Europe, and possibly the oldest in the world (Jerzmańska & Raczyński 1991). Based on the size and structure of the scales in contemporary vendace (*C. albula*) and European whitefish (*C. lavaretus*) it was ascertained that the examined remains show the closest similarity to European whitefish (Jerzmańska & Raczyński 1991). In the present study, we analyzed mtDNA from the vertebrae found at the archeological site.

Material and methods

The skeletal remains analysed in this study (specimen W-G 59/5; Fig. 2) were among the best preserved materials found in the interglacial lacustrine deposits in Wola Grzymalina, Poland.

DNA extraction

The method of DNA extraction used in the study was previously described by Ciesielski *et al*.

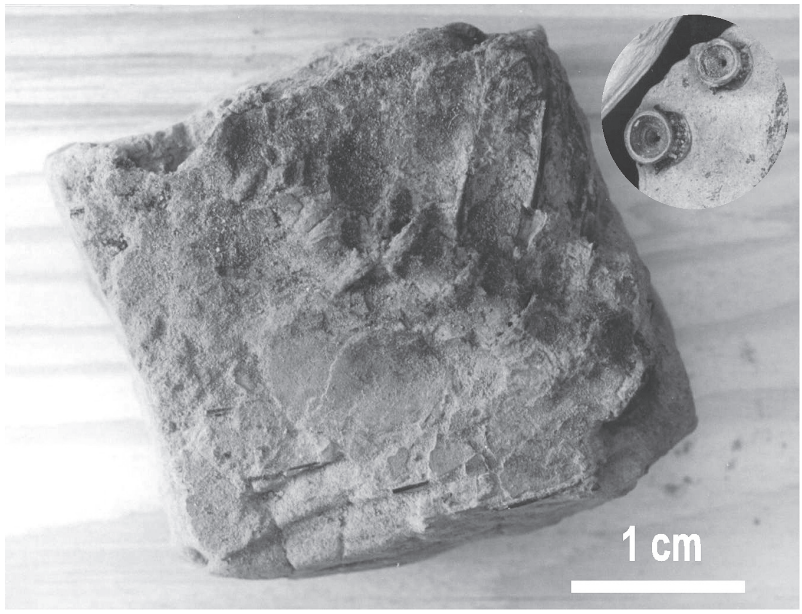

Fig. 2. Specimen W-G 59/5: posterior portion of the skeleton on a piece of laminated diatomite. Two isolated vertebrae are shown within the circle.

(2002). Before DNA extraction, the bone was soaked in sodium hypochlorite solution for surface decontamination, then rinsed several times in ethanol. The vertebrae were sanded with a fine sand paper, and approximately 0.2 g of a bone powder was added to 0.5 M EDTA (pH 8.0) in order to decalcify the sample. After 90 h of incubation with EDTA, the decalcified bone powder was transferred into a tube, then 500 μl of lysis buffer (100 mM Tris-HCl; 10 mM EDTA; pH 8.0, 0.5 mg of proteinase K, and 0.25 mg of dithiothreitol) were added. Then the sample was incubated at 55 °C for 3 h. The aqueous phase was extracted twice with an equal volume of phenol, and once with chloroform–isoamyl alcohol solution (24:1). The upper phase was transferred into another tube containing 0.1 volume of 3 M sodium acetate solution. The DNA was precipitated with 1 volume of isopropanol for 15 min and then the sample was centrifuged at 12 000 g for 15 min to obtain a DNA pellet. The pellet was washed with 70% ethanol, centrifuged again (12 000 g for 15 min), and air dried. Finally the DNA pellet was resuspended in 40 μl of sterile water.

Inhibitors of the polymerase chain reaction (PCR) were removed in the last step of DNA extraction by using GenElute PCR Purification Kit (Sigma). The procedure followed instructions given by the manufacturer for eluting PCR products.

PCR amplification

Primer pairs were either chosen from the literature (Bernatchez *et al*. 1992, Bernatchez & Danzmann 1993, Redenbach & Taylor 1999) or developed in this study to amplify short yet variable regions of coregonid mtDNA (Table 1), with the aim to maximize ancient DNA recovery while allowing the detection of taxonomic differences.

Double stranded PCR amplifications were performed in 50 μl reaction volumes containing 2 units of *Taq* DNA polymerase (Promega, Wisconsin, USA), 5 μl reaction buffer (500 mM KCl, pH 8.5; Triton X-100), 20 pmol of each primer (MWG-BIOTECH, Ebersberg, Germany), 2.5 mM $MgCl_2$, 500 of μM dATP, dCTP, dGTP, and dTTP, and 2 μl of DNA extract. DNA was amplified using a Perkin Elmer 9600 thermal cycler (PE-Applied Biosystems, California, USA) beginning with preliminary denaturation at 95 °C for 5 min. The amplification cycle consisted of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 45 s for a total of 35 cycles, ending with a final elongation step at 72 °C for 3 min. Initial PCR prod-

ucts were reamplified (35 cycles) in the same thermal conditions.

The PCR products were separated by electrophoresis on 1.5% agarose gel, using TBE buffer (0.5M EDTA, pH 8.0). Ethidium bromide (0.1 mg ml^{-1}) was added to the gel and the PCR product was visualised with UV light. Molecular weight marker ϕX 174/*Hinf* I was used.

DNA sequencing

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) at the Institute of Biochemistry and Biophysics in Warsaw, Poland. Approximately 104 base pairs from *ND1* gene were determined for both strands, and about 90bp of mtDNA control region (*CR*), was sequenced from one strand. The nucleotide sequence of a fragment of mitochondrial *ND1* gene was deposited in GenBank under Accession number AF521962.

The authenticity of sequences is supported by a number of factors. The entire DNA extraction procedure was organised following strict standard rules to avoid PCR contamination. The preparation of bone samples, DNA extraction and PCR amplifications were replicated by another laboratory. Thus, the results for the ancient DNA sample were based on repeated analysis derived from two independently processed DNA extracts. Preparation of buffers and PCR set-up was performed in a separate laboratory. All buffers and the water used were autoclaved, in all cases aerosol resistant pipette tips were used to aliquot ingredients. All laboratories were UV illuminated for those periods of time when they were not used. Contamination was monitored through the use of PCR control blanks, and consistent results were obtained for repeated extractions and amplifications. Negative controls were included with each set of amplifications.

Sequence analysis

Correlations among the mtDNA sequences as a result of their shared phylogenetic history, were inferred by a Bayesian analysis using Markov chain Monte Carlo (MCMC) method (Huelsenbeck *et al*. 2000, Huelsenbeck & Bollback 2001). Bayesian inference implemented by using MCMC allows performing analyses without assuming that the phylogenetic history is known without error. The question of phylogenetic relationships of a species group is addressed on all possible trees (τ_i), weighted by the probability that each tree is correct. One can suppose, for example, that each possible phylogenetic tree is either consistent with (*c)*, or inconsistent with (*n)* an evolutionary hypho-

Table 1. Oligonucleotides used for the amplification of the mtDNA control region (*CR*) and mitochondrial gene coding for NADH dehydrogenase subunit I (*ND1*).

Designation	Sequence 5´–3´	Region of mtDNA	Expected product size (bp)	Reference
L	CCACTAGCTCCCAAAGCTA	*CR*	320	Bernatchez *et al.* 1992
H2	CGTTGGTCGGTTCTTAC			Bernatchez & Danzmann 1993
L5[v]	ACAACTTTGGCACCGACAATCCTA	*CR*	87,183,279	This study
H	ACTTTCTAGGGTCCATC			Bernatchez *et al.* 1992
D1	TCTGTGAAGTCAAATGGGGCAC	*ND1*	184	Redenbach & Taylor 1999
C2	CTACGAAGTAAGCCTAGGCCTT			This study
D2	CGAGCAATCAGATGCTTTC	*ND1*	104	This study
C2	CTACGAAGTAAGCCTAGGCCTT			This study

[v] the primer set L5:H produces a ladder of bands in *Coregonus* mtDNA, which are referred as to the number of tandem repeats in the repeat stretch (Brzuzan 2000).

thesis, *X*. Then, the overall probability that the hypothesis is correct will be the sum of the posterior probabilities of trees consistent with the hyphothesis, $f(\tau_c|X)$. The sum of the posterior probabilities of all trees, i.e. $f(\tau_c|X)$ and $f(\tau_n|X)$, will be 1.

In our study, the question concerns the taxonomic position of the fish whose skeletal remains were examined. One hypothesis was that studied vertebrae were from European whitefish, whereas the other considered them as from vendace. The probability that the *lavaretus* hypothesis is correct was the sum of the posterior probabilities of a reconstruction that places the sample W-G 59/5 into one clade with contemporary *C. lavaretus*. Consequently, in the calculation of overall probability of the *albula* hypothesis, only those trees were considered which contained the clade of the ancient sample and *C. albula*. The phylogeny was based on mtDNA sequences sampled from the specimen W-G 59/5, and contemporary European whitefish (Lake Łebsko, Poland) and vendace (Lake Hańcza, Poland). The trees were rooted using rainbow trout, *Oncorhynchus mykiss*, as the outgroup. We examined the hypotheses by approximating the posterior probabilities of the trees, separately for *ND1* and *CR* fragments, using the program MrBAYES (Huelsenbeck & Ronquist 2001). In the MCMC settings we made all trees *a priori* equally probable. The HKY85 (Hasegawa *et al*. 1985) model of DNA substitution was assumed with site-specific bycodon (*ND1* fragment) or Γ (*CR* fragment) rate variation (Yang 1994). Both models allow a different rate of transitions, transversions, different stationary nucleotide frequencies, and among site rate variation. Four Markov chains were run simultanously for 50 000 generations, sampling the chains every 10 generations. We discarded the states of the chains that were sampled before generation 3000.

Results and discussion

The method of DNA extraction we used in two independent laboratories yielded a sufficient amount of amplifiable DNA. Polymerase chain reactions (PCRs) on extracts from the vertebrae were succesful only using primers that targeted sequences not longer than about 100bp. Positive results were obtained for two pieces of ancient mtDNA: a 104bp fragment of *ND1* gene (D2: C2 primer set; Table 1), and a 87bp *CR* fragment (L5:H; Table 1). In either case, the initial PCR products (35 cycles) were not detectable by electrophoresis, but reamplification (another 35 cycles) resulted in a distinct banding pattern. Reamplified blank trials for DNA extraction did not yield any DNA product. An independently replicated 104bp fragment of *ND1* gene was the same as the sequence determined by the other laboratory. However, the independent amplification of the *CR* region did not give a reproducible product; the sequence contained many ambiguous positions and data from this sample were not used.

The sequences of amplified products, *ND1* and *CR*, from the sample W-G 59/5 were aligned with those of contemporary European whitefish and vendace (Fig. 3). The ancient nucleotide sequence of a 102bp fragment of mitochondrial *ND1* gene matched the contemporary sequence of European whitefish and differed from that of vendace by four substitutions. Ambiguous nucleotides were seen at nine positions throughout the *CR* fragment, nonetheless the sequence was different from that of vendace by eight additional changes (Fig. 3). The results of BLAST searches of GenBank's library were most consistent with the *lavaretus* hypothesis (data not shown).

Ambiguous sequences may indicate the presence of contamination, which complicates the interpretation of the results (Pääbo *et al*. 1990). In the study of the other fragment, *ND1*, however, no indication for DNA contamination was present. Detection of ambiguous nucleotides in the *CR* sequence may be expected if one amplifies DNA strands with sequences that occur in an array, i.e. are tandemly repeated, and have similar length but differ at some positions at their overlap. The mtDNA sequence of the primer binding site for L5 includes a block of 9 nucleotides (5´-GGCACCGAC-3´) that has been proposed as a possible candidate binding site for the transcription factor (Shedlock *et al*. 1992). During evolution this control element was multiplied in *Coregonus* mtDNA; the control region sequences may contain 3, 4 or 5 of these regula-

A: ND1 (102bp)

```
              Y  E  V  S  L  G  L  I  L  L  S  V  I  I  F  T  G
W-G 59/5      TACGAAGTAAGCCTAGGCCTTATCTTACTTAGCGTGATTATTTTCACAGGG  51
C. lavaretus ..................................................
C. albula    ................................C...........G..C

              G  F  T  L  Q  T  F  N  V  A  Q  E  S  I  W  L  L
W-G 59/5      GGTTTTACACTTCAGACTTTCAATGTTGCTCAAGAAAGCATCTGATTGCTC 102
C. lavaretus ..................................................
C. albula    ...........C....................................
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B: Control region (87bp)

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W-G 59/5      ACNTTGGCACCGACAATCNTATCATGAAGNCCACTCCTGATNGAAATATNN  51
C. lavaretus ..T.............C..........G...........T.......GT
C. albula    ..T............C.C.G......G.G...........TA...G--AT
                            *   *      *             *   ***
W-G 59/5      GCNGGNGTAGCNTAAGTAAAGCATAACACTGAAGAT 87
C. lavaretus ..T..C.....T.....................
C. albula    ..T..C.....T.....................C.
                                              *
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Fig. 3. Comparisons between the aligned sequences of the fragment of the *ND1* gene (**A**) and control region (**B**) obtained from ancient sample W-G 59/5 and contemporary European whitefish (*C. lavaretus*) and vendace (*C. albula*). (**A**) The ancient *ND1* sequence matched the sequence of *C. lavaretus* and differed from that of *C. albula* by four substitutions. Each nucleotide change occurred in a third position of a respective amino acid codon. The ancient mtDNA *ND1* sequence is deposited in GenBank under the accession number AF521962. (**B**) The ancient control region sequence had nine unknown bases (N), but differed from that of *C. albula* by eight additional changes (asterisks).

tory motifs, depending on the number of repeats in an array (Reed *et al*. 1998, Brzuzan 2000). It has been shown that the size of the repeats in European whitefish varies from 81bp to 104bp, and that sequences of the repeats may differ by small indels, transitions, and transversions (Brzuzan 2000). The sequencing results for the *CR* fragment (Fig. 3) suggest that PCR product obtained with the primer pair L5:H might have contained more than one type of repeat. Further studies, including cloning of the PCR products to determine the homogeneity of the starting template (Handt *et al*. 1994), followed by competitive PCR assays for quantitation of the amount of mtDNA for different fragment lengths (Jehaes *et al*. 2001), with subsequent DNA sequencing should resolve whether this was the case.

The lack of variation between modern-day samples and the ancient samples within a 102-bp *ND1* fragment (Fig. 3) is striking given the age of the fossil. However, this result would be phylogenetically sensible if one showed that potentially variable sites were located outside the short stretch of *ND1* gene studied here. In a molecular phylogeographic study of Redenbach and Taylor (1999) on Arctic grayling inhabiting Russian and North American waters, complete nucleotide sequences of the *ND1* mtDNA have been analyzed, which defined 11 haplotypes. The haplotype sequences differed from one another by several substitutions, however within the corresponding 102bp fragment of the *ND1* gene, the variability was confined to only one single substitution recorded in one haplotype. Bearing in mind that all analyzed grayling haplotypes could have diverged 500 000–2 400 000 years before present, this implies that the lack of variability between modern and ancient whitefish *ND1* sequences is probable.

The main question of the study concerned the taxonomic position of the fish whose skeletal remains were examined. Therefore, we performed a Bayesian analysis of the two mtDNA segments, *ND1* and *CR*, to find their most probable phylogeny. Figure 4 summarizes the results of the analysis. The posterior probability was spread out over three reconstructions. The tree with the largest posterior probability was (*O.*

mykiss, (*C. albula,* (*C. lavaretus,* W-G59/5))), and the posterior probability of this tree was $f(\tau|X) = 0.65$ after analysis of *ND1* fragment and $f(\tau|X) = 0.52$ after analysis of *CR* fragment (Fig. 4). Because posterior probabilities of a reconstruction that placed the sample W-G 59/5 into one clade with extant *C. albula,* i.e. (*O. mykiss,* (*C. lavaretus,* (*C. albula,* W-G59/5))), were only $f(\tau|ND1) = 0.18$ and $f(\tau|CR) = 0.19$), on average, it is three times more likely that the remains found at the site W-G 59/5 were from European whitefish than from vendace. The average posterior probability of a *lavaretus* reconstruction was also three times higher when compared to the reconstruction that involved a contemporary pair of European whitefish and vendace in the clade. Thus, our study provides molecular evidence for the *lavaretus* hypothesis and supports the previous assignment based on the size and structure of the excavated scales compared with contemporary European whitefish (Jerzmańska & Raczyński 1991).

Preliminary studies of mammalian bones preserved in permafrost deposits have shown that genetic information can be retrieved from material aged more than 60 000 years B.P., beyond the limit of radiocarbon dating (Greenwood *et al*. 1999, Vila *et al*. 2001, Barnes *et al*. 2002). The preservation of approximately 100bp fragment in vertebrae that are about 500 000 years old, that have not been preserved in typical permafrost, and that contained sufficient DNA to enable direct DNA sequencing after amplification, may be partly attributed to specific features of the diatomite matrix of the fossil fish. This sedimentary substance, composed of the siliceous skeletons of diatoms, may have formed favourable anoxic conditions for the mtDNA to "survive" for thousands of years. Some experimental observations indicate that DNA half-life may be very long in sediments, particularly if the DNA remains inside the cell remnants (Rollo 1998).

The results obtained from the specimen W-G 59/5 suggest that some other samples may be amenable to molecular analysis. To obtain a more complete picture of the European whitefish assemblage described from the deposits in Wola Grzymalina (Jerzmańska & Raczyński 1991), additional samples should be analysed. The excellent preservation of the European whitefish specimen analysed in this study suggests a potential for studying genetic variation within the species over thousands of years.

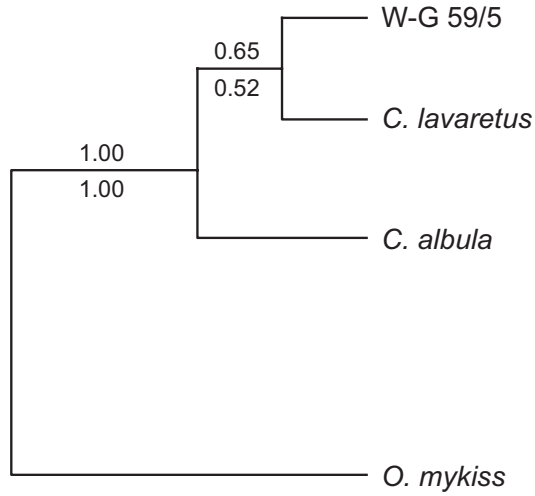

Fig. 4. The tree with the maximum posterior probability for the analyses of the mtDNA sequences of ancient (W-G 59/5) and contemporary *C. lavaretus* and *C. albula*. The numbers at the interior nodes represent the posterior probability that the clade is correct; the numbers above the branches show the values for *ND1* gene fragment, whereas the numbers below the branches show the values for *CR* fragment. The posterior probabilities of clades were approximated with the program MrBAYES (Huelsenbeck & Ronquist 2001). The tree was rooted using rainbow trout (*Oncorhynchus mykiss*) as the outgroup.

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