J Mol Evol (1992) 34:85–90

Journal of Molecular Evolution

Comparative Study of Two Protein-Coding Regions of Mitochondrial DNA from Three Endemic Sculpins (Cottoidei) of Lake Baikal

M.A. Grachev, S.Ja. Slobodyanyuk, N.G. Kholodilov, S.P. Fyodorov, S.I. Belikov, D.Yu. Sherbakov, V.G. Sideleva, A.A. Zubin, and V.V. Kharchenko

Limnological Institute, Siberian Division of the Academy of Sciences of USSR, Irkutsk

Summary. Two protein-coding regions (cytochrome b, ATPase 8, and part of ATPase 6) from mitochondrial DNA of *Cottus kessleri, Cottocomephorus grewingki,* and *Cottocomephorus inermis*—Baikalian endemic sculpins—were amplified via polymerase chain reaction, and sequenced. Two novel primers—L8352 (5′-TAAAGATTGGTGAC TCCCAACCACC) and H8773 (5′-GTAGGGAGT AAGCCCAATATGTT)—were used for the latter region. Phylogenies suggested by sequence divergence of the genes of ATPases appeared to be different from those computed from data for cytochrome b. The time of species branching was estimated as 1–2 million years (Myr) on the basis of merged sequences. Hence, members of the Baikalian cottoid species flock are much more distant from each other than members of the cichlid fish flocks of the great lakes of Africa (0.2 Myr). Topology of the phylogenetic tree does not contradict the relationships derived from morphological data. However, genetic distances suggest that *C. grewingki* and *C. inermis* are not sister species, contrary to general belief.

Key words: Mitochondrial DNA – PCR – Cottoid fishes – Baikal

Introduction

Lake Baikal (East Siberia) is believed to have existed for 30–50 million years (Myr) (Logachov and Florensov 1978). It is inhabited by many hundreds of endemic organisms, among them more than 20 sculpins—Cottoidei (Sideleva 1982). Detailed studies of molecular phylogeny and dating of the most important geological events in the rift valley of Baikal may shed new light on the mechanisms by which flocks of endemic species appear in closed aquatic ecosystems. Species flocks of Lake Baikal have been a subject of great interest to many biologists for decades (Brooks 1950), but it is only now that phylogenetic relationships within such flocks can be studied by DNA sequencing methods.

Phylogenetic relationships between organisms are most often studied by analysis of their mitochondrial DNAs (mtDNAs) (Billington and Hebert 1988; Chang et al. 1989). The rate of mtDNA evolution is relatively high—some 2% of base substitutions per 1 Myr, 5–8 times higher than that of genomic DNA (Brown et al. 1979).

Two methods are generally used to compare sequences of mtDNAs. One is based on comparison of restriction fragment patterns. The second is direct sequencing of polymerase chain reaction (PCR) products (Kocher and White 1989). Direct sequencing is more informative, but requires selection of conservative targets for oligonucleotide primers.

We started the present studies in 1989, when almost no data on mtDNA of fishes were available. Therefore we had to select by partial sequencing a region of mtDNA suitable for amplification. A 607-bp-long fragment of *Cottus kessleri* coding for lysine tRNA, ATPase 8, and part of the ATPase 6 gene was sequenced by the Maxam–Gilbert method to yield the necessary information for successful PCR. We also used the primers proposed by Kocher et al. (1989) to amplify a part of the gene of mitochondrial cytochrome b. PCR, sequencing, and likelihood maximization treatment of the sequences of the

Offprint requests to: M.A. Grachev

abovementioned genes gave phylogenetic trees and estimates of the times of branching for three Baikalian cottoid species: *C. kessleri, Cottocomephorus grewingki,* and *Cottocomephorus inermis.*

Materials and Methods

Isolation of mtDNA. Eggs (2–3 g) were extracted from fishes and placed immediately into cold (4°C) Buffer A (0.2 M sucrose, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The preparations were stored on ice for no more than 48 h prior to treatment in the laboratory. Essentially, the method of rapid isolation of mtDNA was based upon the procedure of Jones et al. (1988). All operations were performed at 0–2°C. Eggs were homogenized in 10 volumes of Buffer A in a plastic vessel with a Teflon pestle. The homogenate was twice centrifuged at 15,000 × *g* (15 min). The supernatant was centrifuged for 30 min at 12,000 × *g*. The supernatant was discarded and the precipitate (mitochondria) was resuspended in 400 μl of Buffer B (100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM EDTA). Mitochondria were either lysed immediately by addition of SDS to 0.2% and Proteinase K to 100 μg/ml (10 min incubation at 37°C), or stored at −20°C. The lysate was extracted once with phenol and twice with chloroform. DNA was precipitated with two volumes of ethanol, dissolved in 500 μl of TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA), and treated with RNase A (10 μg/ml, 1 h at 25°C). After the treatment, the solution was twice extracted with chloroform, and DNA was precipitated with two volumes of ethanol and dissolved in 50 μl of TE.

Restriction and Electrophoresis. EcoRI and HindIII restriction endonucleases were used. Five microliters of stock mtDNA solution was used for each reaction. Electrophoresis of DNA fragments was performed in 0.4% or 0.8% agarose gels, at 15 V/cm in 1× TPE (Sambrook et al. 1989).

Cloning in Plasmid Vector. The 3.3-kb HindIII fragment of mtDNA of *C. kessleri* was cut out of the gel slab, eluted, and cloned in plasmid vector pUC18 as described by Sambrook et al. (1989).

Maxam–Gilbert Sequencing. Cottus kessleri mtDNA or cloned fragments of mtDNA were treated with an appropriate restriction endonuclease (EcoRI), ^{32}P-labeled using Klenow DNA-polymerase I (Sambrook et al. 1989), and sequencing reactions performed according to Maxam and Gilbert with acetone precipitation as described by Baram and Grachev (1985). The sequencing gel was 8% or 4% polyacrylamide, 7 M urea, 1× TBE. The gels were exposed to x-ray film for 48 h.

PCR Amplification. PCR amplifications were performed in 50-μl volumes of 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.2 mg/ml bovine serum albumin (Fraction 5, Serva), 3 mM (for the ATPase 8 region of mtDNA) or 1.5 mM (for the cytochrome b region) $MgCl_2$, 0.3 mM of each of the four dNTPs, 0.24 μM primers, with 2 units of Taq polymerase, and 50–100 ng of target DNA. Thirty-five cycles of amplification were performed according to the following program: 70 s at 94°C (melting), 100 s at 55°C (annealing), and 120 s at 72°C (polymerization) using a thermocycler from Lina Co. (Angarsk, USSR). The primers used for the ATPase 8 gene were L8352, 5′TAAAGATTGGTGACT CCCAACCACC; H8773.1, 5′GTAGGGAAGTAAGCCCAATA TGTT; and H8773.2, 5′AGGTAATAAACTAATAGGTT; those for the cytochrome B gene were L14724 (Meyer et al. 1990) and H15149 (Kocher et al. 1989). The four-digit numbers refer to the positions of the 3′ ends of the primers in human DNA; L and H refer to the light and the heavy chain, respectively. LPCR sequencing was performed according to Murray (1989); an appropriate ^{32}P-labeled oligonucleotide was taken as primer. Ten to twenty cycles of PCR under the abovementioned conditions were performed with ddNTPs in 20-μl reaction mixtures containing preamplified gene fragments.

Analysis of Sequences. Optimal tree topologies were computed using the maximum likelihood method with the DNAMLK program of the PHYLIP package (version 3.3; Felsenstein 1990) on

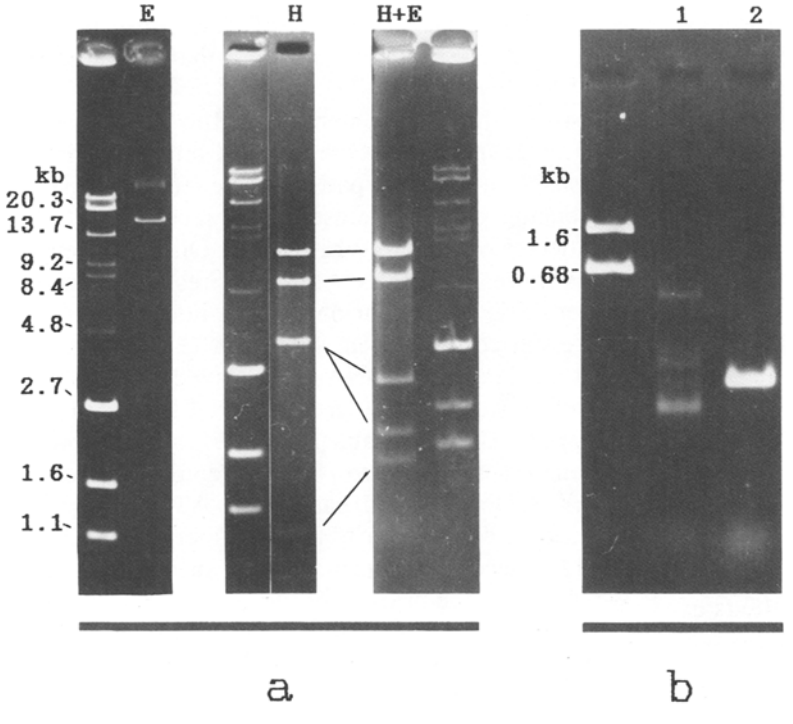

Fig. 1. **a** Electrophoretic patterns of the restriction fragments of *Cottus kessleri* mtDNA; 1% agarose gel, 1× TPE,15 V/cm. E, digestion by EcoRI; H, by HindIII; H+E, by EcoRI and HindIII. **b** Electrophoresis of the ATPase region of mtDNA amplification products; 1.5% agarose gel, 1× TPE, 15 V/cm, 3-μl aliquots of reaction mixtures. (1) L8352 + H8773.2. (2) L8352 + H8773.1.

a Micro-VAX computer. The branching pattern was confirmed using the DNABOOT program of the abovementioned package with 150 replications and several random number seeds. *Xenopus laevis* mtDNA sequences were used as outgroups.

Results and Discussion

Mitochondrial DNA of *C. kessleri* was cleaved by EcoRI (Fig. 1a). Linear DNA thus obtained was ^{32}P-labeled at both ends with Klenow DNA polymerase I and [α-^{32}P]ATP, and the product cleaved with BspR1. Figure 2 shows a part of a Maxam–Gilbert sequencing gel for the longer of the two radioactive fragments obtained. The 230-bp-long sequence obtained was aligned to the known mtDNA sequence of *X. laevis* (Roe et al. 1985) and found to be highly homologous to a region ranging from the 3′ end of the gene of lysine tRNA through a complete reading frame for mtATPase 8 and part of mtATPase 6 (Fig. 3, positions 76–305 in the sequence for *C. kessleri*). The latter two genes overlap over 10 bp in *X. laevis*. It is interesting that the length of a similar overlapping region in *C. kessleri* is exactly the same, whereas the two genes overlap over 40 bp in bovine, and over 46 bp in human mtDNA (Roe et al. 1985).

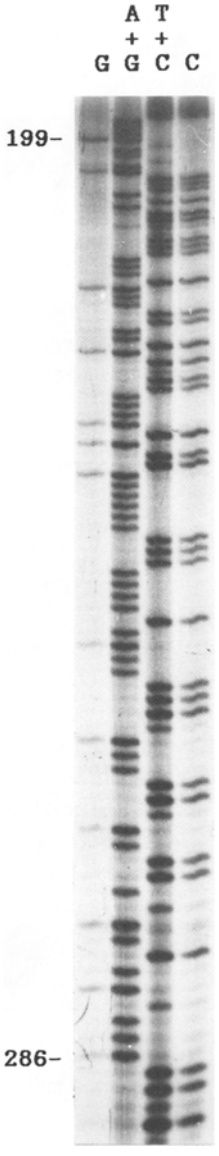

Fig. 2. Part of a Maxam–Gilbert sequencing gel of the longest radioactive fragment obtained from a *Cottus kessleri* circular mtDNA by digestion with EcoRI followed by treatment with Klenow DNA-polymerase I combined with [α-^{32}P]ATP and TTP, and cleavage with BspR1.

Attempts to run PCR with oligonucleotide primers L8352 (sequence derived from *C. kessleri* mtDNA) and H8773.2, complementary to a conserved part of *X. laevis* ATPase 6 gene (Fig. 3, positions 10,280–10,300 of *X. laevis*), failed (Fig. 1b). Therefore we cloned the 3.3-kb Hind3 fragment of *C. kessleri* mtDNA carrying the EcoRI site (Fig. 1a) in a pUC18 plasmid and sequenced it by the Maxam–Gilbert procedure over a total length of 607 bp including the abovementioned 230-bp fragment (Fig. 3). On this basis we chose the primer H8773.1. Together with L8352, it served to amplify the 417-bp-long mtDNA fragments of *C. kessleri* and other Baikalian cottoids (Fig. 1b). The fragments of the *C. grewingki* and *C. inermis* mtDNAs were sequenced by linear polymerase chain reaction (LPCR) according to Murray (1989). The three sequences thus obtained are shown in Fig. 4. These sequences differ from each other at 23 sites, and only by a single amino acid substitution (Table 1).

Primers L14724 and H15149, complementary to the gene for cytochrome b proposed by Kocher et al. (1989), we used to amplify a 382-bp-long fragment in order to obtain a link with the real time scale proposed for a wide variety of taxa (e.g., Brown et al. 1979; Kocher et al. 1989). These sequences were obtained for the three cottoid species by means of LPCR (see Materials and Methods) and also subjected to analysis (Fig. 4). Base substitutions occur at 26 sites; the proportion of transversions is somewhat higher than that found for ATPases (Table 1). The most important difference is the higher rate of amino acid substitutions (four vs one). This finding does not contradict evidence implying lower rates of amino acid substitutions in fishes compared to other vertebrates (Kocher et al. 1989). Substitutions

Table 1. Numbers of base substitutions in pairs of species (above the diagonal, transitions; below the diagonal, transversions in ATPase 8 + 6/cytochrome b regions)

	C. kessleri	*C. grewingki*	*C. inermis*
C. kessleri	–	15/12	11/11
C. grewingki	5/7	–	10/11
C. inermis	5/5	1/7	–

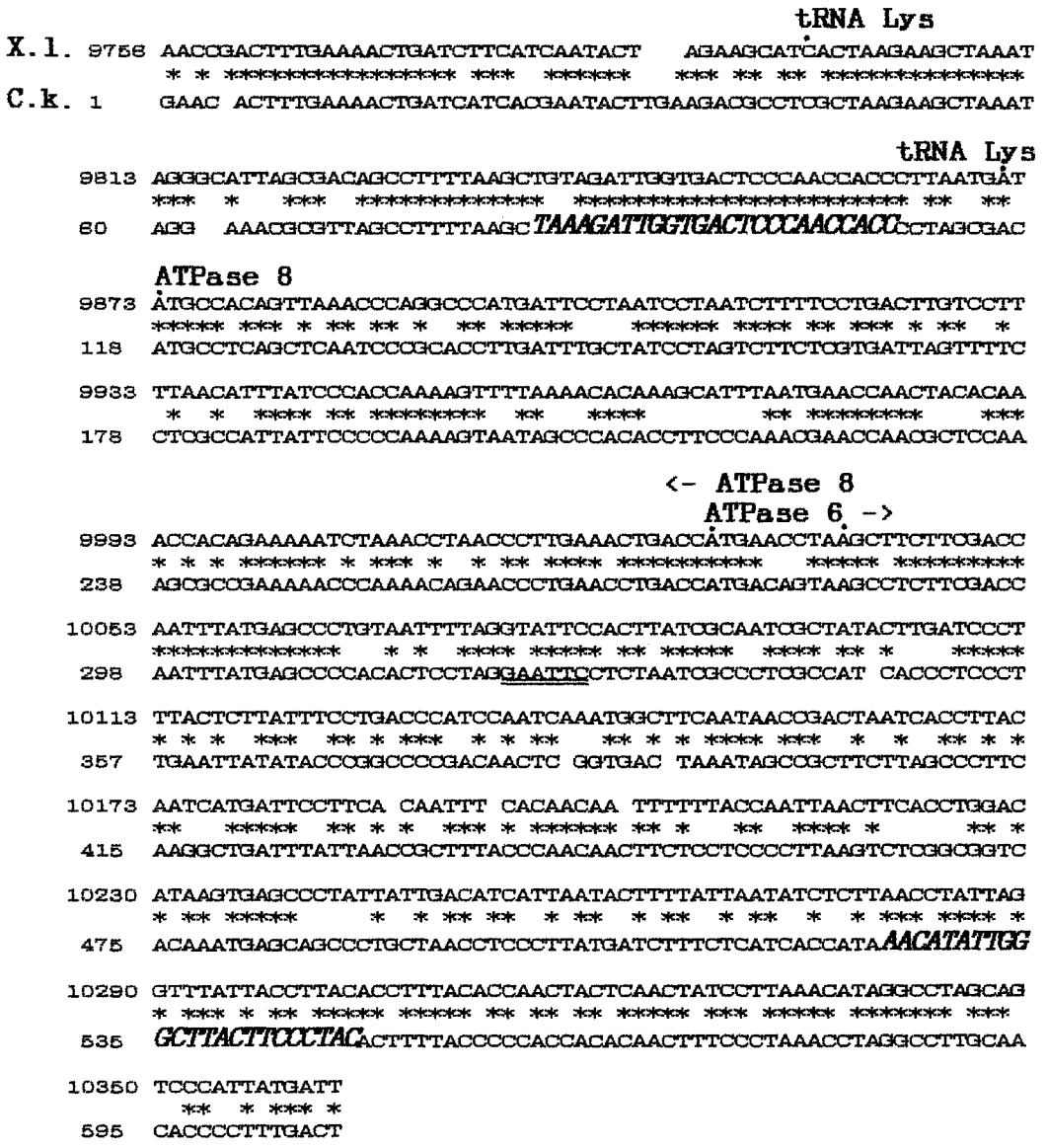

Fig. 3. Sequence of a 607-bp fragment of *Cottus kessleri* mtDNA (L strand) aligned with *Xenopus laevis* mtDNA. EcoRI site is underlined; bold letters, sites interacting with the primers L8352 and H8773; asterisks indicate identical positions.

in cottoid fish mtDNA mostly occur at the sites identified by Kocher et al. (1989) for cichlids.

It is generally believed that the sequence of mitochondrial cytochrome b is very conservative and changes slower than that of other proteins, among them mtATPase. Therefore, the greater difference between the amino acid sequences of the cytochrome b of cottoid fishes compared with the sequence difference in their mtATPases is somewhat unusual. However, comparison of the sequences of cottoid mtDNA with those of cichlid fishes (Fig. 4) exhibits a difference typical of other distantly related

Table 2. Morphoecological features of three Baikalian sculpins (Taliev 1955; Sideleva 1982)

	C. kessleri	*C. grewingki*	*C. inermis*
Habitat	Near-shore, depth to 200 m	Upper part of slope, depth 20–300 m	Upper part of slope, depth 20–500 m
Ecological group	Bottom	Near bottom-pelagic	Near bottom-pelagic
Egg size	0.8 mm	1.8 mm	1.5 mm
Fecundity	Up to 10,000 eggs	Up to 2,000 eggs	Up to 3,500 eggs
Color	Greenish	Brown-red	Silvery
Form of head	Round	Compressed from sides	Compressed from sides
Number and form of gillrakers	4–6, hill-form	16–19, hair-form	11–19, hair-form

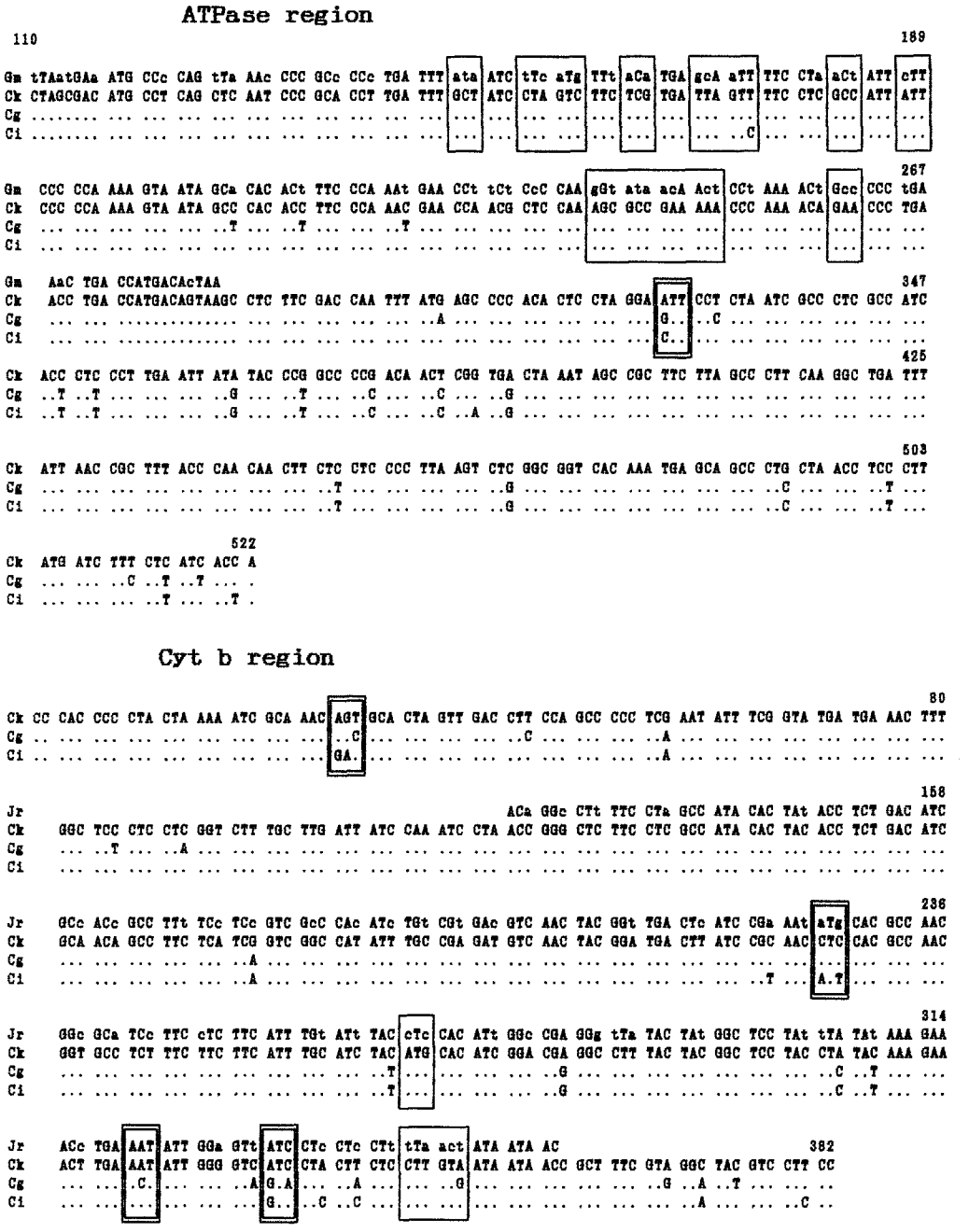

Fig. 4. Alignment of cottoid mtDNA ATPase and cytochrome b regions to mtDNA of Atlantic cod (ATPase 8 gene, Johansen et al. 1989) and to mtDNA of cichlid fishes (cytochrome b region, Kocher et al. 1989); C.k., *Cottus kessleri*; C.g., *Cottocomephorus grewingki*; C.i., *Cottocomephorus inermis*; G.m., *Gadus morhua* (Atlantic cod); *J.r., Julidochromis regani* (a cichlid fish). Dots in cottoid fish sequences indicate bases identical to those of *C. kessleri* mtDNA. Small letters in G.m. and J.r. sequences indicate mismatches with any of the cottoid sequences. Simple boxes outline codons where base mismatches result in amino acid substitutions between G.m. (or J.r.) and all cottoids. Double boxes indicate the sites where amino acid substitutions take place within the cottoid group. Three fishes gave identical sequences in the case of *C. kessleri*, two fishes in the case of *C. grewingki*, and only one specimen of *C. inermis* was analyzed.

species. Comparison with mtDNA of *Gadus morhua* (Atlantic cod) also suggests that cytochrome b is more conservative than mtATPases.

One may speculate on the apparent greater conservativeness of ATPases compared to cytochrome b in cottoid fishes. Presumably, these molecules allow certain numbers of amino acid substitutions that do not affect functions. The fact that this number is smaller for cytochromes b compared to ATPases in cottoid fishes may be due to specific pressure of selection characteristic of habitats where speciation takes place under very slowly changing conditions, as happens in Lake Baikal.

Evolutionary trees were built for the three Baikalian cottoid species using the two sequenced regions separately and the merged sequences (Fig.

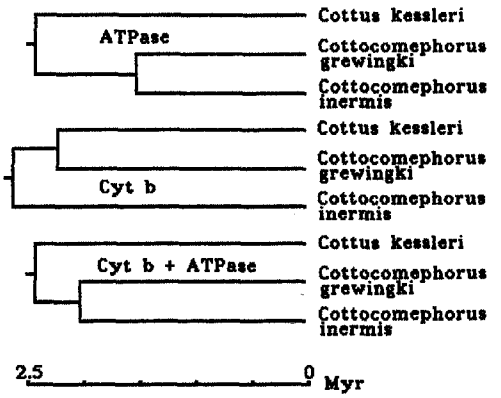

Fig. 5. Phylogenetic analysis of the ATPase region and cytochrome b sequences of three cottoid species. Quantitative data are summarized in Table 1. The lowest tree is the result of merged sequences analysis. Time scale was derived from Kocher et al. (1989, see text).

5). These trees were chosen as having maximum likelihoods using the DNAMLK program of the PHYLIP package, version 3.3 (Felsenstein 1990). Estimation of the times of branching (Fig. 5) under the assumption that the rate of Baikalian cottoid mtDNA evolution was approximately the same as that of mtDNA of mammals (Brown et al. 1979) suggests that separation of the three species happened some 1–2 Myr ago. This time period is much longer than that of the branching of cichlid fishes (0.2 Myr) from Lake Victoria and Lake Malawi (Meyer et al. 1990). It corresponds to the beginning of the deep-water stage of the development of Lake Baikal when the lake existed as at least two separate water basins (Logachov and Florensov 1978).

Morphological evidence suggested that *C. grewingki* and *C. inermis* are sister species, whereas *C. kessleri* belongs to a different subfamily (Taliev 1955; Sideleva 1982). Table 2 gives an outline of a few morphological and ecological features in support of this viewpoint. However, rooted trees constructed on the basis of the sequences of cytochrome b suggest that *C. grewingki* and *C. inermis* are very distant from each other and are not sister species (Fig. 5). *Cottus inermis* appears to be even closer to *C. kessleri* than to *C. grewingki.* Similar trees computed for the sequences of the genes of ATPases, on the contrary, suggest that *C. grewingki* and *C. inermis* are closer to each other than to *C. kessleri.* Merged sequences suggest a tree similar to that inferred from the sequences of ATPases (Fig. 5). However, species grouping places the branching point into the interval where the exact branching order could hardly be resolved within the confidence interval estimated using the bootstrap method (Felsenstein 1985; Hasegawa and Kishino 1989). The only conclusion is that the three species have branched (if they are monophyletic) at the time when haplochromeines of Lake Victoria separated from those of Lake Malawi (Meyer et al. 1990). Hence, cottoid fish of Lake Baikal are much more ancient than cichlid fish of the great lakes of Africa. Presumably, this is due to the fact that Baikal is also much older (Brooks 1950).

References

Baram GI, Grachev SA (1985) Use of lithium perchlorate for the isolation and analysis of oligo- and polynucleotides. Bioorg Chem (USSR) 11:1420–1424

Billington N, Hebert PDN (1988) Mitochondrial DNA variation in Great Lakes walleye (*Stizostedion vitreum*) populations. Can J Fish Aquat Sci 45:643–654

Brooks JL (1950) Speciation in ancient lakes. Q Rev Biol 25: 131–176

Brown WM, George M, Wilson AC (1979) Rapid evolution of animal mitochondrial DNA. Proc Natl Acad Sci USA 76: 1967–1971

Chang H, Wang D, Ajala FJ (1989) Mitochondrial DNA evolution in the *Drosophila nasuta* subgroup of species. J Mol Evol 28:337–348

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Felsenstein J (1990) PHYLIP manual version 3.3. University Herbarium, University of California, Berkeley

Hasegawa M, Kishino H (1989) Confidence limits on the maximum-likelihood estimate of the hominoid tree from mitochondrial-DNA sequences. Evolution 43:672–677

Johansen S, Guddal PH, Johansen T (1989) Organization of the mitochondrial genome of Atlantic cod, *Gadus morhua.* Nucleic Acids Res 18:411–419

Jones CS, Tegelstrom H, Latchman DS, Berry RJ (1988) An improved rapid method for mitochondrial DNA isolation suitable for use in the study of closely related populations. Biochem Genet 26:83–88

Kocher TD, Thomas WK, Meyer A, Edwards SV, Paabo S, Villablanca FX, Wilson AC (1989) Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc Natl Acad Sci USA 86:6196–6200

Kocher TD, White TJ (1989) Evolutionary analysis via PCR. In: Erlich HA (ed) PCR technology. Stockton Press, New York, pp 137–147

Logachev NA, Florensov NA (1978) The Baikal system of rift valleys. Tectonophysics 45:1–13

Meyer A, Kocher TD, Basasibwaki P, Wilson AC (1990) Monophyletic origin of Lake Victoria cichlid fishes suggested by mitochondrial DNA sequences. Nature 347:550–553

Murray V (1989) Improved double-stranded DNA sequencing using linear polymerase chain reaction. Nucleic Acids Res 17: 8889

Roe BA, Ma DP, Wilson RK, Wong GFH (1985) The complete nucleotide sequence of the *Xenopus laevis* mitochondrial DNA genome. J Biol Chem 260:9759–9774

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor NY

Sideleva VG (1982) Seismo-sensor system and ecology of Baikalian sculpins (Cottoidei). Novosibirsk, Nauka

Taliev DN (1955) Baikalian sculpins (Cottoidei). Izd Akad Nauk SSSR, Moscow

Resubmitted June 10, 1991/Accepted June 20, 1991