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Evolutionary history of freshwater sculpins, genus *Cottus* (Teleostei; Cottidae) and related taxa, as inferred from mitochondrial DNA phylogeny

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Abstract

The freshwater sculpins, genus *Cottus* (Teleostei; Cottidae), comprise bottom-dwelling fishes that exhibit various life-history styles, having radiated throughout Northern Hemisphere freshwater habitats. The phylogenetic relationships among *Cottus* and related taxa were estimated from mitochondrial DNA 12S rRNA and control region (CR) sequences, the freshwater sculpins examined falling into five lineages (A–E). Lineage A consisted of *Trachidermus fasciatus* and *C. kazika*, both having a catadromous life-history. The remaining species (lineages B–E) spawn in freshwater habitats regardless of life-history (amphidromous, lacustrine or fluvial), suggesting that the various life-history types post-dated a common ancestor of lineages B–E. Molecular clock estimates suggested a Pliocene–Pleistocene radiation (or Miocene–Pliocene from the alternative clock) of lineages B–E. In eastern Eurasia, speciation with life-history changes to amphidromous or fluvial styles has apparently occurred independently in some lineages, as a general pattern. Mitochondrial DNA CR phylogeny showed the monophyletic Baikalian cottoids (Cottoidei) to be nested within *Cottus* and *Trachidermus*, suggesting that the former ecologically and morphologically divergent cottoids may have originated from a single lineage which invaded the ancient lake.
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1. Introduction

The sculpins (Scorpaeniformes; Cottoidea) are one of the largest and most diverse teleostean groups, containing more than 300 species classified into nine families (e.g., Nelson, 1994; Yabe, 1985). Although most sculpins live in Northern Hemisphere cool-temperate and subarctic marine habitats, some inhabit freshwater habitats (freshwater sculpins). Except for the cottoids (Cottoidei) endemic to Lake Baikal, Siberia (Baikalian cottoids), the freshwater sculpins comprise *Cottus*, *Mesocottus*, *Myoxocephalus*, and

Trachidermus species (Family Cottidae) (Goto, 1990; Lee et al., 1980; Scott and Crossman, 1973; Sideleva, 2001). Among these genera, *Cottus* is the most specious, with about 40 species, compared with *Mesocottus* (1 species), *Myoxocephalus* (3), and *Trachidermus* (1) (Berg, 1949; Lee et al., 1980; Nakabo, 2002; Scott and Crossman, 1973). The freshwater sculpins, especially *Cottus* and related genera, are regarded as a unique group, derived from marine sculpins, which has invaded freshwater habitats.

Cottus includes bottom-dwelling fishes widely distributed in Northern Hemisphere freshwater habitats, the Pacific slope of North America, and eastern Eurasia, including the Japanese Archipelago, being the most species-rich region (Berg, 1949; Lee et al., 1980; Nakabo, 2002; Reshetnikov et al., 1997; Scott and Crossman, 1973). Based on morphological and zoogeographical data, the

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common ancestor of *Cottus* is supposed to have originated in those regions (Sideleva, 1994). Because *Cottus* species exhibit a high degree of species' diversity and morphological variability, as found in other widespread cold-water-adapted freshwater fishes, many studies have been conducted on their diversity in Europe and North America (Kinziger and Wood, 2003; Koli, 1969; Lyons, 1990; Riffel and Schreiber, 1998; Strauss, 1986, 1989).

The diversity and evolution of the *Cottus* fishes have been variously argued in Japan (Goto, 1990, 2001b; Kurawaka, 1976; Nakabo, 2002). Of the *Cottus* species in Japanese freshwaters, the *C. pollux* complex is known to be polytypic, with the most problematic entities (Goto, 1998, 2001a,b; Mizuno and Niwa, 1961; Nakabo, 2002). The complex is considered to include three ecological types, conveniently named *C. pollux* small-egg type, *C. pollux* middle-egg type, and *C. pollux* large-egg type (see Goto, 1998), all being morphologically similar but genetically and ecologically distinguishable from each other (Fujii, 2001; Kurawaka, 1976; Nakabo, 2002; Okazaki and Kobayashi, 1992; Okazaki et al., 1994; Shimizu et al., 1994). According to the genetic evidence for the existence of reproductive isolation between them (Okazaki and Kobayashi, 1992; Okazaki et al., 1994), each of the three ecological types is currently regarded as a distinct species although the taxonomy is complicated (Goto, 1998, 2001a; Nakabo, 2002). Moreover, from genetic studies, populations of *C. pollux* small-egg type are closely related to *C. reinii*, regarded as Lake Biwa endemic (Okazaki and Kobayashi, 1992).

The majority of *Cottus* species have a fluvial or lacustrine life-history, living entirely in freshwater (Lee et al., 1980; Scott and Crossman, 1973). However, in eastern Eurasia, including the Japanese Archipelago, a number of *Cottus* species exhibit various life-history styles, including catadromous, amphidromous, lacustrine, and fluvial (Byeon et al., 1995; Goto, 1990; Goto and Nakano, 1993; Kurawaka, 1976; Shimizu et al., 1994). On the basis of osteological and myological studies, a catadromous sculpin, *Trachidermus fasciatus*, has been proposed as the sister group of *Cottus* (Yabe, 1985). The evolution of life-history styles of freshwater sculpins in this area, therefore, has been investigated by ecological and genetical studies, with reference to speciation processes (Goto, 1990; Goto and Andoh, 1990; Kurawaka, 1976; Mizuno, 1963; Okumura and Goto, 1996). On the basis of early ontogeny between sibling species, fluvial life-history species are considered to have evolved from ancestral amphidromous species, following adaptation to an upstream habitat by increasing egg size and consequent truncation of the larval period (Goto, 1990; Mizuno, 1963). These patterns are generally accepted as evolutionary trends in the freshwater sculpins. However, such studies have been conducted only between closely related species, there being many *Cottus* species for which phylogenetic relationships are little known.

The endemic cottoids in Lake Baikal (Baikalian cottoids) represent a morphologically and ecologically divergent group, which has possibly radiated within the Lake and is currently classified into 3 families, 12 genera, and 33 species (Sideleva, 2001). The origin of this highly divergent group has been investigated morphologically over a long period (e.g., Berg, 1949; Sideleva, 1994; Taliev, 1955). Several recent molecular studies have also been conducted in the hope of revealing phylogenetic relationships (Grachev et al., 1992; Hunt et al., 1997; Kiril'chik and Slobodyanyuk, 1997; Kiril'chik et al., 1995; Kontula et al., 2003; Nishida et al., 1999; Slobodyanyuk et al., 1995). In these studies, the Baikalian cottoids were represented as closely related to each other, suggesting relatively recent radiation (5–2 million years ago), despite their remarkable morphological and ecological divergence. Moreover, Kontula et al. (2003) showed that the Baikalian cottoids had a monophyletic origin, in addition to a close relationship with some *Cottus* species. However, the phylogenetic position of the Baikalian cottoids relative to *Cottus* species and related taxa is not yet known.

Despite the many studies of morphological and ecological evolution of *Cottus* and related taxa, including the Baikalian cottoids, comprehensive studies of the evolutionary history of these fishes are rare, as most works have focused primarily on either a single species, sibling species or species' flocks. To understand the evolution of biological diversity, a reconstruction of the phylogeny of the organisms of interest is fundamental (Brooks and McLennan, 1991). Nevertheless, no study to date has provided a widely accepted phylogenetic perspective for the genus *Cottus* and related taxa, using either molecular or morphological characters.

The purpose of this study was to provide a phylogenetic hypothesis for freshwater sculpins, including *Cottus* and related taxa, based on the nucleotide sequences of mitochondrial DNA (mtDNA) 12S rRNA and control region (CR). The evolution of their life-history styles is proposed based on the phylogeny obtained. In addition, CR sequences of Baikalian cottoids available on database (Kontula et al., 2003) were used together with the present data to assess the phylogenetic position of this highly divergent group within/among *Cottus* and related taxa. Finally, the evolutionary history of freshwater sculpins is discussed.

2. Materials and methods

2.1. Fish samples and DNA extraction

Nineteen individuals, representing 16 species of freshwater sculpins (14 *Cottus*, 1 *Leocottus*, and 1 *Trachidermus*) were collected from Japan, Korea, Russia, Europe, and North America, almost all species of

Cottus and *Trachidermus* in Eurasia being represented. Sampling localities and other details for each species are presented in Table 1. In order to examine intraspecific variation in *C. poecilopus*, which is distributed throughout northern Eurasia (Berg, 1949; Reshetnikov et al., 1997), specimens from Europe and Russia were used in the analyses. Because *C. nozawae*, an endemic Japanese species, include some intraspecific genetic groups (Okumura and Goto, 1996; Yokoyama and Goto, 2002), two individuals were used as representatives of those groups. In this study, each of the three taxonomic entities included in the *C. pollux* complex (see above) was regarded as a distinct species. Nomenclature of the Baikalian cottoids follows Sideleva

(2001). A marine sculpin, *Leptocottus armatus*, regarded as the sister group of *Cottus* and *Trachidermus* by osteological study (Yabe, 1985), was also included in this study. *Myoxocephalus stelleri* was collected from Hokkaido, Japan.

Samples for DNA extraction comprised fin clips or muscle tissues preserved in ethanol, or frozen muscle tissues (below −30 °C). Fin clips or tissues were digested with proteinase K in TNES-urea buffer (Asahida et al., 1996). DNA was purified with standard phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1) extractions. Ethanol precipitated DNA was rinsed with 70% ethanol and resuspended in TE buffer.

Table 1
Sampling locality and accession numbers of freshwater cottoids and out-group examined in this study

Sample	Sampling locality	Accession No.	
		12S rRNA	Control region
Cottoidea			
Cottidae			
Cottus kazika	Gakko R. (Honshu, Japan)	AB188175	AB188157
Cottus pollux large-egg type	Kinu R. (Honshu, Japan)	AB188176	AB188158
Cottus pollux middle-egg type	Amanogawa R. (Hokkaido, Japan)	AB188177	AB188159
Cottus pollux small-egg type	Inabe R. (Honshu, Japan)	AB188178	AB188160
Cottus reinii	Chinai R. (L. Biwa, Honshu, Japan)	AB188179	AB188161
Cottus nozawae	Otofuke R. (Hokkaido, Japan)	AB188180	AB059335[a]
	Otaru R. (Honshu, Japan)	AB188181	AB059348[a]
Cottus amblystomopsis	Tokotan R. (Hokkaido, Japan)	AB188182	AB188162
Cottus hangiongensis	Moheji R. (Hokkaido, Japan)	AB188183	AB188163
Cottus czerskii	Sedanka R. (Vladivostok, Russia)	AB188184	AB059350[a]
Cottus poecilopus	Manoma R. (Amur R. system, Russia)	AB188185	AB188164
	Porovinka R. (Amur R. system, Russia)	AB188186	AB188165
	Duna Stream (Odra R. system, Poland)	AB188187	AB188166
Cottus koreanus	Kongnim R. (Namhan R. system, Korea)	AB188188	AB188167
Cottus gobio	Radunia R. (Vistula R. system, Poland)	AB188189	AB188168
Cottus cognatus	Anadyr R. (Russia)	AB188190	AB188169
Cottus aleuticus	L. Karluck (Alaska, USA)	AB188191	AB188170
Leocottus kesslerii	L. Baikal (Russia)	AB188192	AB188171
Trachidermus fasciatus	Fukanomi R. (Kyushu, Japan)	AB188193	AB188172
Leptocottus armatus	Camp Pendleton (California, USA)	AB188194	AB188173
Myoxocephalus stelleri	L. Akkeshi (Hokkaido, Japan)	AB188195	AB188174
Cottus bairdii	L. Michigan (USA)[b]	—	AY116394[b]
Cottus cognatus	L. Michigan (USA)[b]	—	AY116396[b]
Cottus sibericus	Olkha R. (Russia)[b]	—	AY116398[b]
Batrachocottus nikolskii	L. Baikal (Russia)[b]	—	AY116389[b]
Cottocomephorus grewingkii	L. Baikal (Russia)[b]	—	AY116390[b]
Paracottus knerii	L. Baikal (Russia)[b]	—	AY116393[b]
Abyssocottidae			
Abyssocottus gibbosus	L. Baikal (Russia)[b]	—	AY116372[b]
Abyssocottus korotneffi	L. Baikal (Russia)[b]	—	AY116373[b]
Asprocottus herzensteini	L. Baikal (Russia)[b]	—	AY116375[b]
Cottinella boulengeri	L. Baikal (Russia)[b]	—	AY116378[b]
Cyphocottus eurystomus	L. Baikal (Russia)[b]	—	AY116380[b]
Limnocottus godlewskii	L. Baikal (Russia)[b]	—	AY116381[b]
Procottus jeittelesii	L. Baikal (Russia)[b]	—	AY116384[b]
Comephoridae			
Comephorus baicalensis	L. Baikal (Russia)[b]	—	AY116386[b]

R., River; L., Lake.
[a] Yokoyama and Goto (2002).
[b] Kontula et al. (2003).

2.2. DNA amplification and sequencing

Polymerase chain reaction (PCR) amplification for entire CR and part of tRNAs was carried out using the primers L-Thr and H12Sr5 (Yokoyama and Goto, 2002). The 5′ part of 12S rRNA region was amplified by the PCR using the primers C947m (5′-AAAGCA TAACASTGAAKATG-3′) and H1478m (5′-GAGG GTGACGGGCGGTGTGT-3′). PCR amplifications were performed in a total reaction volume of 25 μl, containing 0.5 U of TaKaRa EX *Taq* (TaKaRa), 2.5 μl of $10 \times EX$ *Taq* buffer (TaKaRa), 2 μl of dNTP mix (2.5 mM each), 0.5 μl of each primer (25 mM), and 1 μl of template DNA. Thermal cycle conditions were as follows: initial denaturation at 94 °C for 1 min; followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 50–58 °C for 1 min, depending on the primer specificity for different samples, and extension at 72 °C for 1 min, with final extension at 72 °C for 5–10 min. PCR products were purified from residual primers and nucleotides by polyethylene glycol precipitation for direct sequencing. Sequencing was performed using PCR primers and internal primers, LCCR and H16498m (Yokoyama and Goto, 2002) for CR, and C1606 (5′-GCTGAAG ATGGCGGTATATA-3′) for 12SrRNA, respectively. All sequencing reactions were prepared with the Thermo Sequenase II dye terminator cycle sequencing kit (Amersham Pharmacia Biotech), according to the manufacturer's directions, and analyzed on a 373S DNA sequencer (Applied Biosystems Inc.). Sequence data were edited and connected using DNASIS (Hitachi Software Engineering Co., Ltd.). The nucleotide sequences have been submitted to the DDBJ database.

2.3. Sequence alignment

Sequences were aligned using CLUSTAL X (Thompson et al., 1997) with default settings and adjusted by eyes. Insertions/deletions and ambiguous alignment positions in the CR were excluded from all taxa in subsequent phylogenetic analyses. The incongruence length difference test (Farris et al., 1995), as implemented in the partition homogeneity test in PAUP* 4.0b10 (Swofford, 2002), was performed to assess statistical confidence for combining the 12S rRNA and CR datasets (12S + CR dataset). De Queiroz's method (de Queiroz, 1993) for evaluating dataset incongruence with a support/conflict criterion of 90% bootstrap support was also used.

2.4. Phylogenetic analysis

Phylogenetic relationships for the 12S + CR dataset were inferred by maximum-parsimony (MP), maximum likelihood (ML), and neighbor-joining (NJ) methods using PAUP* 4.0b10 (Swofford, 2002). MP analysis was performed with heuristic searches (TBR branch swapping and random addition of taxa). Hierarchical likelihood ratio tests to determine the most appropriate substitution model for the datasets were performed using ModelTest version 3.06 (Posada and Crandall, 1998). ML analysis was computed based on the HKY85 (Hasegawa et al., 1985) model of nucleotide substitution with gamma distribution shape parameter (G) and proportion of invariable positions (I), using the heuristic algorithm. NJ analyses were done with the distance matrix calculated using HKY85 + G + I. To estimate statistical support for the branching patterns, 1000 bootstrap (Felsenstein, 1985) replications were performed for the MP (TBR branch swapping and random addition of taxa) and NJ analyses. Bayesian inference of phylogeny was performed using MrBayes v3.0 (Huelsenbeck and Ronquist, 2001). HKY + G + I model of molecular evolution was used. The Metropolis-coupled Markov chain Monte Carlo process was set for four chains to run simultaneously for 1,000,000 generations with sampling trees every 100 generations. Posterior probabilities for clades were estimated by a majority-rule consensus tree. The lower 10% of trees (1000 trees) were discarded in the computation of majority-rule consensus tree. In all analyses, sequences of a marine sculpin, *Myoxocephalus stelleri*, was used as out-group. Statistical tests for the phylogenetic hypotheses were conducted using the Shimodaira–Hasegawa test (Shimodaira and Hasegawa, 1999) for the ML tree.

To estimate the evolution of life-history styles, the reproductive and early life-history traits were superimposed on the ML tree (12S + CR dataset) using MacClade 3.04 (Maddison and Maddison, 1992).

The molecular clock hypothesis was tested using the branch length test implemented in LINTRE (Takezaki et al., 1995), based on the 12S + CR dataset. The test was applied to the NJ tree constructed using NJBOOT2 implemented in LINTRE (Hasegawa gamma distance option). To construct a linearized tree, we eliminated deviant sequences (at 1% level). Subsequently, a molecular clock-enforced ML tree was constructed as a linearized tree (Nei and Kumar, 2000), using PAUP* 4.0b10 (Swofford, 2002).

To consider the phylogenetic relationships of freshwater sculpins, including Baikalian cottoids and North American species, CR sequences reported by Kontula et al. (2003), which were comparable to the present study, were downloaded from GenBank/DDBJ. According to their phylogenetic inferences (Kontula et al., 2003), we chose CR sequences of freshwater cottoids (representing 3 families, 11 genera, and 13 species (Table 1)), from their dataset as representatives of Baikalian and North American cottoids, in order to compare our data (CR2 dataset). The MP, NJ, ML, and Bayesian analyses were performed with same procedures as 12S + CR dataset.

3. Results

3.1. Sequence variations

The lengths of part of the 12S rRNA resulted in 780–784 bp for all taxa, entire CR and partial tRNAs ranging from 960 to 968 bp for the in-group (*Cottus* and *Trachidermus*) and from 966 to 1086 bp for the out-group. The resulting sequence alignments were 780 and 887 bp for 12S rRNA and CR, respectively. The CR sequences of sculpins had some conserved structures, terminal-associated sequences (TAS), conserved sequence blocks (CSB-D, 2, and 3), and pyrimidine tracts (PY), as reported in other fishes (e.g., Chen et al., 1998; Lee et al., 1995; Takahashi and Goto, 2001). The partition homogeneity test revealed no significant differences between the 12S rRNA and CR sequences ($P = 0.607$). Using bootstrap criterion (>90% bootstrap for MP) as a measure of dataset incongruence, there were no conflicting nodes strongly supported. Given these results and because individual genes lack resolving power, we prefer to combine our data. The CR and 12S rRNA were combined and used as a single set (12S + CR dataset) for the following phylogenetic analyses. Alignment of the CR sequences including Baikalian and North American cottoids (Kontula et al., 2003) resulted in 798 bp length (CR2 dataset).

3.2. Phylogenetic analyses

The appropriate model and parameters for the ML and NJ analyses were estimated by the hierarchical likelihood ratio test. For 12S + CR, the HKY85 + G (0.66) + I (0.49) model was selected. Because the MP, ML, NJ, and Bayesian methods using the 12S + CR dataset (1667 bp) produced almost identical topologies, we represent the ML tree (Fig. 1). All branch lengths were significantly different from zero ($P < 0.01$) in the ML tree, except for the two branches at basal node. These two branches are presented as collapsed relationships in Fig. 1.

In all methods, the freshwater sculpins examined fell into five major lineages, A, B, C, D, and E (Fig. 1). Lineage A comprised *T. fasciatus* and *C. kazika*, being strongly supported in all analyses (100% bootstrap for NJ and MP, and 100% posterior probability for Bayesian). Lineage B consisted of three intraspecific variants of *C. poecilopus* from Poland and the Amur River basin (99 and 95% bootstrap for NJ and MP, respectively, and 100% posterior probability for Bayesian). The HKY + G + I distance (12S + CR) between haplotypes from the Amur River basin was low (0.011), contrasting with relatively high values between haplotypes from Poland and the Amur River basin (0.030–0.037) (Table 2). Lineage C included two species' pairs; *C. amblystomopsis* and *C. nozawae*, and *C. hangiongensis* and *C. koreanus*. Statistical support for the monophyly of each species' pair was moderate to high in all analyses (97–100% bootstrap for NJ, 74–100% boot-

strap for MP and 100% posterior probabilities for Bayesian). Pairwise HKY + G + I distances (12S + CR) within and between the species' pairs were 0.017–0.038 and 0.054–0.062, respectively. The *C. pollux* complex, *C. reinii* and *C. czerskii* comprised lineage D (83% bootstrap for NJ, 81% bootstrap for MP, and 100% posterior probability for Bayesian), *C. czerskii* being the sister of the *C. pollux* complex + *C. reinii*. In lineage D, two taxon pairs, *C. pollux* small-egg type and *C. reinii*, and *C. pollux* large-egg type and *C. pollux* middle-egg type, were supported by high statistical probabilities (97–100% bootstrap for NJ, 91–99% bootstrap for MP, and 100% posterior probabilities for Bayesian). The genetic distance (HKY + G + I for 12S + CR) between *C. pollux* small-egg type and *C. reinii* was quite low (0.009). Monophyly of the *C. pollux* complex + *C. reinii* was also supported by high statistical values (100% bootstrap for NJ, 98% bootstrap for MP and 100% posterior probability for Bayesian). Lineage E consisted of cottoids distributed in Europe (*C. gobio*), Lake Baikal (*Leocottus kesslerii*), and North America (*C. aleuticus* and *C. cognatus*), although the monophyly of them was statistically supported only in NJ (75% bootstrap) and Bayesian (98% posterior probability) analyses.

The relationships among the species within each lineage were well resolved in this dataset, except for lineage E, whereas the interrelationships among the lineages B, C, D, and E were not resolved confidently. The monophyly of the lineages B, C, D, and E was shown in all methods, the branch having length significantly different from zero ($P < 0.01$), but with relatively low support (87% bootstrap for NJ, 53% bootstrap for MP, and 94% posterior probability for Bayesian). Lineage A was inferred to be a sister group with the lineages B, C, D, and E in all methods (53% bootstrap for NJ, 64% bootstrap for MP, and 96% posterior probability for Bayesian). However, likelihood test for interior branch length for the ML tree did not reject a null hypothesis (branch length = zero) for this branch. Therefore, we represented this branch as collapsed (Fig. 1). Because the lineage A included both *T. fasciatus* and *C. kazika* in all tree reconstructions, the genus *Cottus* must be considered polyphyletic. The hypothesis for monophyly of genus *Cottus*, including *Leocottus kesslerii* from Lake Baikal, the generic name having been recently changed from *Cottus* by Sideleva (2001), was clearly rejected by the Shimodaira–Hasegawa test for the ML tree ($P = 0.0002$).

3.3. Life-history evolution

Life-history evolution was traced on the ML tree. Because similar results were obtained using the other trees, only the life-history reconstruction on the ML tree is shown (Fig. 2). The trait of breeding habitat information (Table 3) was mapped on the ML tree (Fig. 2a). Species in lineage A, plus *Leptocottus armatus* and *Myoxocephalus stelleri*, spawn in seawater habitats. On the other hand, all
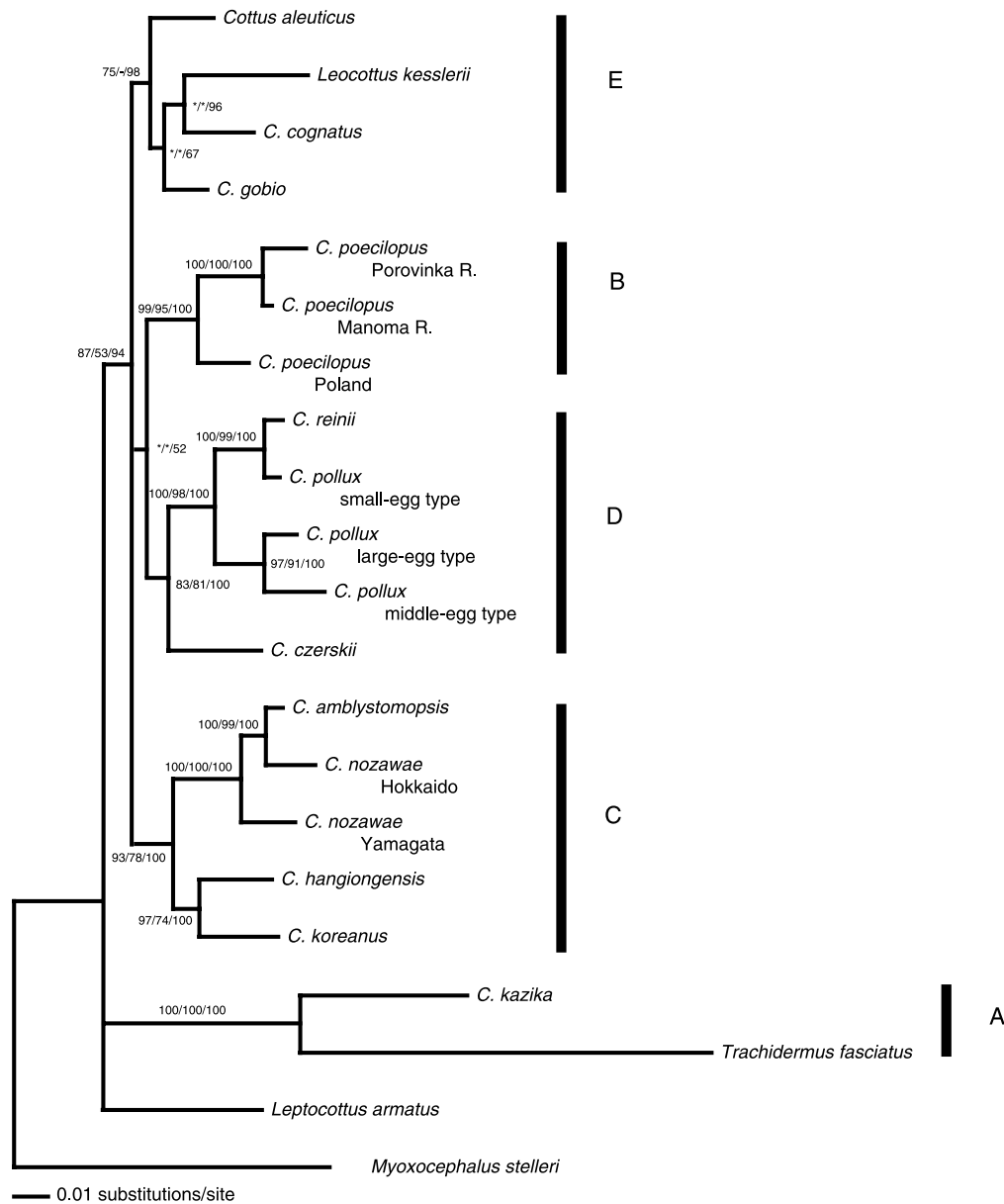
Fig. 1. Maximum likelihood tree representing mitochondrial DNA phylogeny among freshwater cottoids, based on 12S + CR dataset (1667 bp) using HKY85 + G (0.66) + I (0.49) model. Numbers indicates bootstrap values (%) from neighbor-joining (left) and maximum-parsimony (center), and posterior probabilities (%) from Bayesian (right) methods. Nodes marked with dash (-) are present in the method but low statistical support (<50% bootstrap). Nodes marked with asterisk (*) are in conflict with the ML analysis.

of the remaining species spawn in freshwater habitats regardless of life-history type. The analysis indicated that freshwater breeding would be common to all species in lineages B, C, D, and E (Fig. 2a). According to the analysis, an amphidromous life-history would have been derived from a fluvial type in the lineages C, D, and E (Fig. 2b).

3.4. Rate consistency test

In the branch length test applied to the NJ tree (12S + CR dataset) using *Myoxocephalus stelleri* as the out-group, sequences of *T. fasciatus, C. kazika, C. aleuti-cus, C. gobio, C. poecilopus* from Poland, *C. czerskii,* and

C. reinii showed significantly different substitution rates from the average rate at the 1% level. In the reanalysis, using the dataset after eliminating the above seven sequences, the rate appeared homogeneous. The molecular clock-enforced ML tree of the 12S + CR dataset, excluding the above sequences, is shown in Fig. 3.

3.5. Control region phylogeny, including the Baikalian cottoids

The ML, NJ, MP, and Bayesian analyses for the CR2 dataset including the Baikalian and North American cottoids produced almost identical topology to each

Table 2
Sequence differences for the 12S + CR dataset from freshwater cottoids and outgroup

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1 *Cottus aleuticus*	—	0.0507	0.0394	0.0287	0.0577	0.0481	0.0509	0.0502	0.0551	0.0587	0.0544	0.0561	0.0521	0.0518	0.0611	0.0594	0.0484	0.1318	0.2013	0.0703	0.1521
2 *Leocottus kesslerii*	4/1 / *16/16*	—	0.0525	0.0483	0.0668	0.0566	0.0596	0.0717	0.0794	0.0793	0.0723	0.0643	0.0646	0.0653	0.0752	0.0751	0.0698	0.1461	0.2085	0.0864	0.1497
3 *C. cognatus*	5/1 / *17/17*	4/1 / *18/16*	—	0.0307	0.0558	0.0497	0.0508	0.0651	0.0705	0.0716	0.0639	0.0655	0.0536	0.0577	0.0693	0.0665	0.0544	0.1226	0.2167	0.0819	0.1425
4 *C. gobio*	4/1 / *13/5*	4/1 / *15/15*	4/1 / *11/8*	—	0.0509	0.0391	0.0450	0.0479	0.0529	0.0569	0.0484	0.0528	0.0493	0.0507	0.0618	0.0547	0.0520	0.1171	0.1855	0.0702	0.1401
5 *C. poecilopus* Porovinka R.	5/2 / *19/15*	5/2 / *18/22*	7/2 / *15/17*	6/2 / *15/14*	—	0.0371	0.0112	0.0623	0.0687	0.0629	0.0641	0.0667	0.0584	0.0610	0.0634	0.0689	0.0580	0.1426	0.2094	0.0873	0.1469
6 *C. poecilopus* Poland	7/1 / *16/11*	7/1 / *15/18*	9/1 / *14/12*	8/1 / *11/9*	5/2 / *15/6*	—	0.0301	0.0577	0.0667	0.0639	0.0522	0.0530	0.0520	0.0554	0.0587	0.0613	0.0544	0.1285	0.2098	0.0769	0.1344
7 *C. poecilopus* Manoma R.	5/2 / *17/14*	5/2 / *14/22*	6/2 / *14/15*	5/2 / *13/13*	1/1 / *5/3*	10/4 / *16/13*	—	0.0614	0.0686	0.0595	0.0605	0.0621	0.0524	0.0558	0.0573	0.0626	0.0518	0.1394	0.2126	0.0813	0.1401
8 *C. amblystomopsis*	10/3 / *12/12*	10/3 / *17/21*	11/3 / *17/15*	10/3 / *12/10*	11/4 / *15/14*	12/3 / *16/10*	10/4 / *16/13*	—	0.0166	0.0243	0.0542	0.0549	0.0704	0.0709	0.0787	0.0748	0.0620	0.1434	0.2124	0.0931	0.1589
9 *C. nozawae* Hokkaido	9/3 / *14/14*	9/3 / *20/22*	11/3 / *18/17*	11/3 / *14/11*	10/4 / *15/14*	12/3 / *16/10*	11/4 / *19/15*	8/1 / *9/7*	—	0.0323	0.0620	0.0621	0.0787	0.0793	0.0874	0.0824	0.0672	0.1542	0.2116	0.0994	0.1676
10 *C. nozawae* Yamagata	10/3 / *15/14*	10/3 / *19/22*	10/3 / *20/17*	10/3 / *14/14*	10/4 / *19/15*	13/3 / *19/12*	11/4 / *19/20*	8/1 / *6/4*	11/4 / *13/12*	—	0.0561	0.0587	0.0745	0.0730	0.0771	0.0711	0.0628	0.1451	0.2128	0.1019	0.1663
11 *C. hangiongensis*	6/2 / *18/13*	6/2 / *19/23*	6/2 / *21/20*	6/2 / *15/13*	7/2 / *20/15*	9/2 / *15/11*	7/2 / *19/17*	11/4 / *11/13*	11/4 / *14/15*	11/4 / *13/12*	—	0.0382	0.0639	0.0644	0.0690	0.0698	0.0550	0.1253	0.1803	0.0934	0.1529
12 *C. koreanus*	8/1 / *17/15*	9/1 / *15/21*	6/2 / *20/17*	6/2 / *14/18*	6/3 / *20/20*	10/1 / *15/12*	7/2 / *17/19*	13/3 / *13/10*	12/3 / *12/3*	13/3 / *13/12*	8/2 / *10/9*	—	0.0688	0.0703	0.0720	0.0714	0.0578	0.1466	0.2095	0.0863	0.1465
13 *C. reinii*	5/2 / *19/11*	6/2 / *19/18*	6/1 / *20/15*	6/2 / *15/13*	6/3 / *17/15*	7/2 / *17/12*	6/3 / *18/17*	13/10 / *13/10*	17/11 / *17/11*	16/10 / *16/10*	8/3 / *18/15*	5/2 / *14/10*	—	0.0093	0.0321	0.0445	0.0498	0.1237	0.2202	0.0876	0.1470
14 *C. pollux* small-egg type	4/3 / *18/13*	5/3 / *20/19*	5/3 / *19/15*	5/3 / *15/14*	5/4 / *21/14*	7/3 / *17/15*	5/2 / *18/14*	11/4 / *17/18*	11/4 / *20/19*	11/4 / *21/15*	7/4 / *18/17*	9/2 / *20/19*	4/1 / *2/2*	—	0.0306	0.0419	0.0506	0.1233	0.2085	0.0862	0.1513
15 *C. pollux* large-egg type	6/1 / *19/18*	7/1 / *19/18*	7/1 / *24/26*	7/1 / *22/24*	7/5 / *17/8*	7/3 / *18/12*	5/2 / *17/19*	12/2 / *12/11*	11/2 / *11/2*	12/2 / *12/2*	8/2 / *8/2*	9/1 / *20/21*	6/2 / *9/8*	6/2 / *8/7*	—	0.0249	0.0542	0.1336	0.2129	0.0987	0.1583
16 *C. pollux* middle-egg type	5/2 / *19/17*	6/2 / *21/20*	6/2 / *19/18*	6/2 / *17/20*	6/3 / *18/20*	8/2 / *19/17*	5/3 / *18/9*	12/3 / *12/3*	11/3 / *11/3*	11/3 / *11/3*	8/3 / *18/20*	10/2 / *20/18*	6/3 / *14/10*	5/3 / *12/11*	4/1 / *9/6*	—	0.0539	0.1238	0.2054	0.0913	0.1594
17 *C. czerskii*	4/1 / *17/14*	5/1 / *21/22*	10/4 / *18/23*	5/1 / *19/13*	4/2 / *21/10*	6/1 / *21/10*	4/2 / *19/13*	10/3 / *14/17*	10/3 / *16/19*	9/3 / *15/17*	5/2 / *18/15*	7/1 / *18/15*	5/2 / *21/24*	4/3 / *19/26*	5/1 / *22/11*	5/2 / *20/12*	—	0.1306	0.2069	0.0857	0.1393
18 *C. kazika*	18/8 / *26/26*	19/8 / *26/32*	17/8 / *24/26*	18/7 / *22/26*	17/8 / *28/30*	19/8 / *25/24*	17/9 / *26/29*	22/9 / *25/27*	21/9 / *27/29*	21/9 / *27/27*	18/9 / *25/24*	22/8 / *26/27*	19/10 / *24/25*	21/9 / *23/26*	18/8 / *25/28*	18/8 / *24/26*	18/7 / *26/26*	—	0.1582	0.1422	0.2243
19 *T. fasciatus*	19/7 / *38/39*	19/8 / *35/44*	19/8 / *40/41*	20/7 / *33/39*	18/8 / *35/45*	20/8 / *38/40*	18/9 / *36/45*	22/8 / *34/43*	20/8 / *35/44*	21/8 / *35/42*	17/9 / *35/36*	22/8 / *36/40*	20/9 / *38/42*	19/8 / *36/43*	19/7 / *35/46*	19/8 / *34/44*	18/7 / *39/42*	15/6 / *32/36*	—	0.2225	0.2846
20 *Leptocottus armatus*	7/3 / *17/22*	9/4 / *18/27*	10/4 / *18/23*	9/4 / *13/23*	7/5 / *20/26*	9/4 / *17/23*	7/5 / *18/26*	12/6 / *15/29*	12/6 / *17/30*	16/6 / *18/28*	11/5 / *18/27*	11/4 / *16/27*	8/5 / *21/24*	8/6 / *19/26*	9/4 / *21/30*	9/5 / *20/27*	8/3 / *20/26*	18/9 / *23/33*	22/8 / *33/48*	—	0.1506
21 *Myoxocephalus stelleri*	10/5 / *30/42*	9/5 / *27/45*	10/5 / *27/42*	10/5 / *28/40*	10/6 / *29/40*	11/5 / *27/37*	10/6 / *28/39*	15/6 / *27/41*	14/6 / *30/42*	16/6 / *28/42*	11/6 / *29/42*	12/5 / *27/41*	8/6 / *33/39*	9/6 / *34/38*	9/4 / *35/41*	10/5 / *34/41*	10/4 / *29/39*	20/9 / *35/48*	21/9 / *38/59*	12/6 / *24/45*	—

Values above the diagonal are HKY + G + I distance for 12S + CR dataset; below diagonal are number of transitions/transversions for 12SrRNA (upper) and control region (lower-italic), respectively.
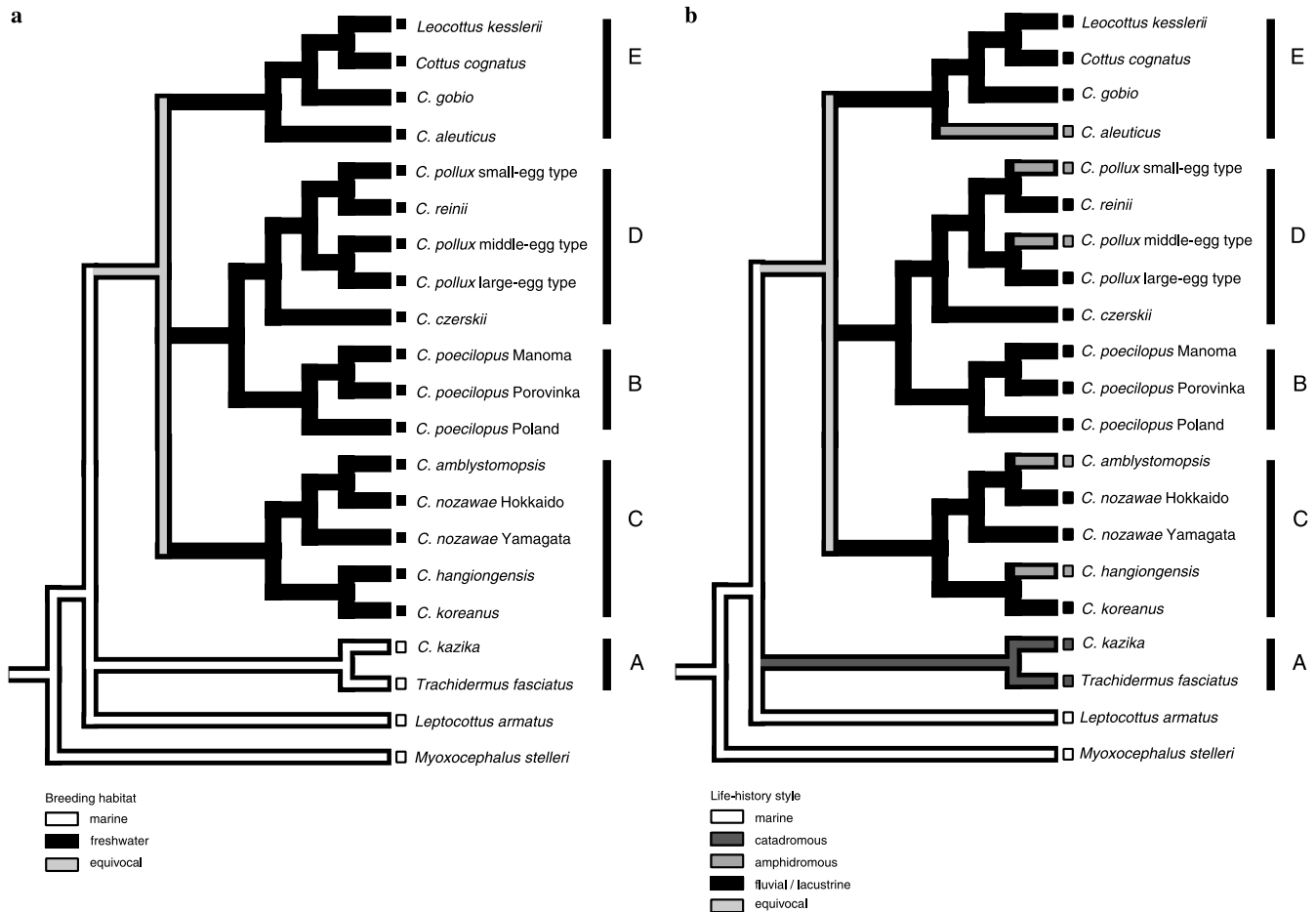
Fig. 2. Life-history evolution reconstructed with maximum parsimony. A maximum-likelihood tree based on 12S + CR dataset was used as a phylogenetic tree. Lineages refer to those presented in Fig. 1. (a) Distribution of breeding habitats onto the proposed tree. White branches indicate lineages which spawn in marine habitats, Black branches representing lineages with freshwater breeding. (b) Evolution of life-history styles traced onto the mitochondrial DNA phylogeny of freshwater cottoids.

other. The freshwater cottoids also fell into lineages A, B, C, and D (Fig. 4), the members of them being identical to those from the 12 + CR dataset, in spite of the addition of sequences from the Baikalian and other cottoids. The lineages A, B, and C were supported by moderate–high statistic values (Fig. 4). The statistic support for the lineage D was low (53% bootstrap for NJ, <50% bootstrap for MP, and 95% posterior probability for Bayesian). The Baikalian cottoids clustered into a monophyletic group in all methods, but this association received poor statistical support. The Baikalian, North American (*C. cognatus*, *C. aleuticus*, and *C. bairdii*), Siberian (*C. sibericus*), and European (*C. gobio*) cottoids comprised a monophyletic group in all methods, but this association was statistically supported in Bayesian analysis only (97% posterior probability).

Interrelationships among the lineages were not resolved confidently same as those from 12S + CR dataset. Likelihood ratio test for interior branch lengths for the ML tree revealed that all branches have lengths significantly different from zero excluding nine branches

($P < 0.01$). The nine branches where the null hypothesis could not be rejected were presented as collapsed in Fig. 4. The monophyly of the lineages B, C, D, and the Baikalian, North American, Siberian, and European cottoids was shown in all methods, the branch length significantly different from zero ($P < 0.01$), but with low support (<50% bootstrap for NJ, 61% bootstrap for MP, and 93% posterior probability).

4. Discussion

4.1. Phylogenetic remarks

The present analyses of the phylogenetic relationships among 14 (mainly East Asian) species of *Cottus* and their relatives, *Leocottus kesslerii* and *T. fasciatus*, revealed that the genus *Cottus* was polyphyletic (Fig. 1).

Excessively fast rates of divergence were found for *T. fasciatus* and *C. kazika* (lineage A). In some animals, a short generation time is considered to be inversely

Table 3
Geographic distribution and life-history styles of freshwater sculpins and out-group taxa included in this study

Species	Geographic distribution	Life-history styles[a]	Breeding habitat	Reference
Cottus kazika	Japanese Archipelago	C	Marine	Kinoshita et al. (1999)
C. pollux large-egg type	Japanese Archipelago	F	Freshwater	Kurawaka (1976)
C. pollux middle-egg type	Japanese Archipelago	A	Freshwater	Shimizu et al. (1994)
C. pollux small-egg type	Japanese Archipelago	A	Freshwater	Kurawaka (1976)
C. reinii	Lake Biwa (Japan)	L	Freshwater	Kurawaka (1976)
C. nozawae	Northern Japan	F	Freshwater	Goto (1990); Goto et al. (2001)
C. amblystomopsis	Hokkaido (Japan), Sakhalin and Primorsky (Russia)	A	Freshwater	Goto (1980, 1990)
C. hangiongensis	Korea, Northern Japan, Primorsky (Russia)	A	Freshwater	Byeon et al. (1995); Goto (1981)
C. czerskii	Far East Russia	F	Freshwater	Berg (1949)
C. poecilopus	Northern Eurasia	F/L	Freshwater	Berg (1949)
C. koreanus	Korea	F	Freshwater	Byeon et al. (1995); Fujii et al. (2005)
C. gobio	Western Siberia, Europe	F/L	Freshwater	Downhower et al. (1990); Wanzenbock et al. (2000)
C. cognatus	North America, Chukotsk (Russia)	F/L	Freshwater	Lee et al. (1980); Scott and Crossman (1973)
C. aleuticus	Pacific slope of North America	A (L/F)	Freshwater	Lee et al. (1980); Brown et al. (1995); Scott and Crossman (1973)
Leocottus kesslerii	Lake Baikal and adjacent basin (Russia)	L/F	Freshwater	Berg (1949)
Trachidermus fasciatus	Southern Japan, coastal slope of Yellow and East China Sea	C	Marine	Takita and Chikamoto (1994)
Leptocottus armatus	Pacific coast of North America	M/B	Marine	Jones (1962)
Myoxocephalus stelleri	Northern Pacific	M/B	Marine	Nakabo (2002)

[a] C, catadromous; A, amphidromous; L, lacustrine; F, fluvial; M, marine; B, brackish.

related to substitution rate (Martin and Palumbi, 1993). Almost all *T. fasciatus* individuals mature at 1 year, no 2 year fish have been found in Japan (Takita and Chikamoto, 1994). In contrast, Japanese *Cottus* species, excluding *C. kazika*, are known to mature at 2–3 years (Goto, 1989, 1990). Differences in maturity ages, which affect generation times, may have resulted in the faster rate in *T. fasciatus* compared with other freshwater sculpins. Excessive fast rates in *T. fasciatus* and *C. kazika* might lead to a problem in phylogenetic inference known as long branch attraction (Hendy and Penny, 1989). We analyzed almost all the freshwater cottoids suggested to be relatives of *Cottus* species (Yabe, 1985). Taxonomic sampling in this study was thought to be reasonable at this point. *T. fasciatus* and *C. kazika*, included in lineage A, have a catadromous life-history (Kinoshita et al., 1999; Takeshita et al., 1997; Takita and Chikamoto, 1994), which is a unique trait in freshwater cottoids. From an ecological point of view, their relationship as indicated by the present study seems to be reasonable. Further analysis using other genetic markers including nuclear loci is needed to resolve the dynamics of molecular evolution and the phylogenetic position of lineage A. We also need to explore other related taxa including marine sculpins to resolve the basal relationships of freshwater cottoids more confidently.

The Korean fluvial sculpin had been recorded in the name of *C. poecilopus* (Byeon et al., 1995; Jeon, 1987). Recent morphological studies of *C. poecilopus*, including type specimens, revealed that the Korean fluvial sculpin represented neither *C. poecilopus* nor any other *Cottus* species described from Far East Russia, Japan or Korea (Fujii et al., 2005). Fujii et al. (2005) described the Korean fluvial sculpin as a new species, *C. koreanus*. The type locality of *C. poecilopus* is in the Vistula basin, Slovenia (Heckel, 1837; Kottelat, 1997). If the *C. poecilopus* sample from Poland (Odra River basin) used in this study can be regarded as a true representative of *C. poecilopus*, lineage B would represent the *C. poecilopus* lineage in our result (Fig. 1). *Cottus koreanus* was not closely related to the *C. poecilopus* lineage (lineage B), but to *C. hangiongensis* (lineage C). The hypothesis for monophyly of *C. poecilopus* and *C. koreanus* was clearly rejected by Shimodaira–Hasegawa test for the ML tree based on 12S + CR dataset ($P = 0.0035$). The molecular phylogeny presented here supports the Fujii et al.'s view (2005) and reveals the phylogenetic position of a newly described species, word only *C. koreanus*.

The relationship between the *C. amblystomopsis* and *C. nozawae* species' pair was paraphyletic, corresponding to previous studies (Goto and Andoh, 1990; Okumura and Goto, 1996), in which aspects of this complex relationship have already been discussed. The haplotype of *C. reinii*, an endemic species in Lake Biwa, Japan, is genetically similar to that of small-egg type *C. pollux* (HKY + G + I distance, 0.009; Table 2), the value being regarded as indicative of intraspecific variation. From the mtDNA phylogeny, the two samples apparently
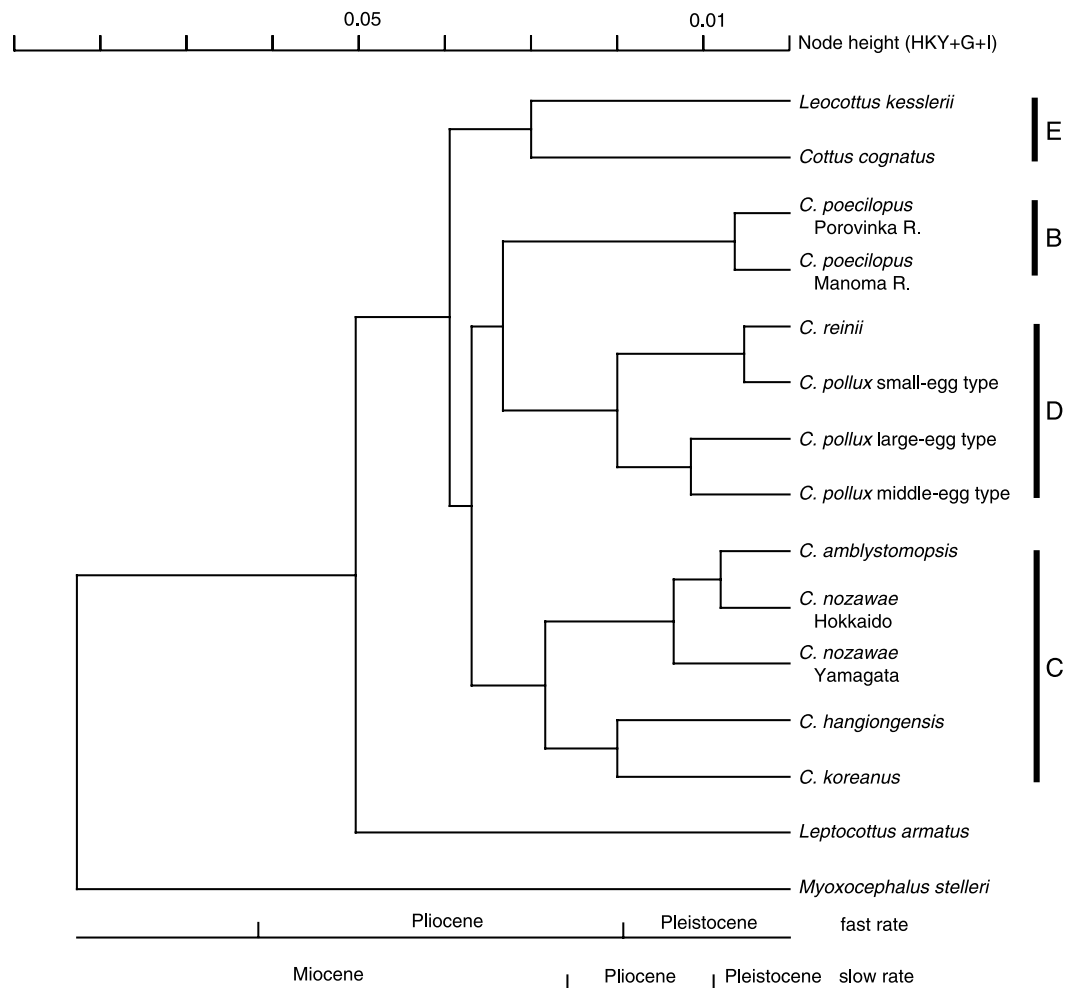
Fig. 3. Maximum-likelihood tree with molecular clock enforcement for freshwater cottoids based on 12S + CR dataset, excluding deviant sequences (see text). Scale bars above and below the tree indicate HKY + G + I distance between sequences and the time scales calibrated by fast (2.4% per Myr) and slow (1.0% per Myr) rates, respectively. Lineages refer to those presented in Fig. 1.

represent genetic variants within a single species rather than distinct species, although slight differences in morphological and ecological traits were found between them (Fujii, 2001).

It is suggested that the Baikalian, North American, and European cottoids would comprise a single lineage (lineage E), although the statistical support was relatively weak (Fig. 1). The mtDNA CR-based phylogeny of freshwater cottoids suggested that the Baikalian cottoids could be clustered into a monophyletic group and nested within *Cottus* and *Trachidermus*, but with poor support (Fig. 4). Kontula et al. (2003) showed the strong evidence for monophyly of the Baikalian cottoids. Moreover, a clade comprising either *C. bairdii* + *C. cognatus* or *C. gobio* + *C. sibericus* would be the sister lineage of the Baikalian cottoids (Kiril'chik and Slobodyanyuk, 1997; Kontula et al., 2003). The lineage E in this study corresponded to those studies. Judging from these results, we could consider the possibility of the monophyly of the Baikalian, North American, European, and Siberian cottoids. Further analysis, using much informative markers and samples of North American *Cottus* species, should clarify the relationships among the species of this divergent lineage.

Interrelationships among the lineages A, B, C, D, and E were less resolved, although the monophyly of the lineages B–E was estimated (Fig. 1). This suggests that the dataset used in this study would be enough to resolve the relationships among closely related species, but have insufficient phylogenetic information to resolve deep phylogeny. Alternatively, short lengths of basal branches and poor resolution among them may be due to the simultaneous diversification of each lineage from the common ancestor, indicating the necessity of analysis using much informative dataset.

4.2. Life-history evolution

In this analysis, we postulate the monophyly of lineages B–E, according to the phylogenetic trees estimated
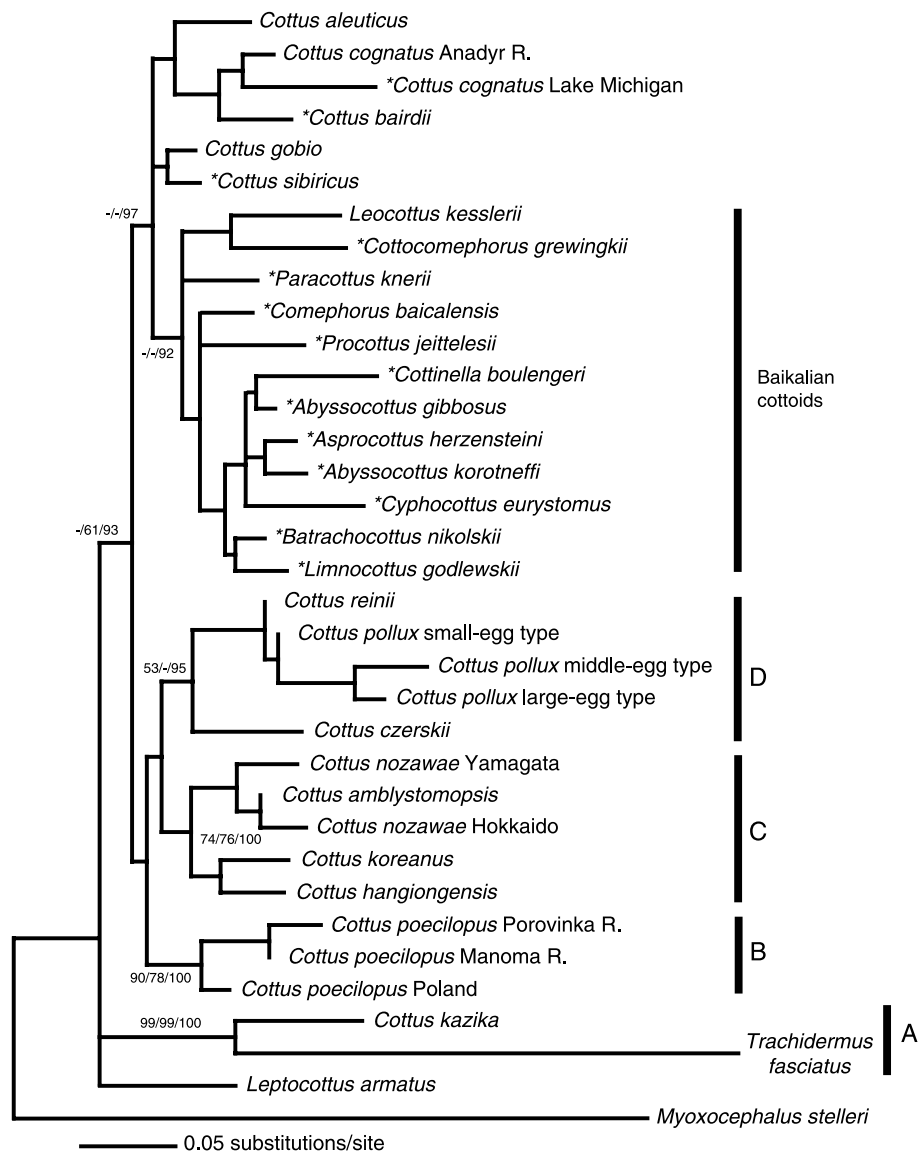
Fig. 4. Maximum-likelihood tree among freshwater cottoids, including Baikalian and North American species (Kontula et al., 2003), based on CR2 dataset (798 bp) using the HKY + G + I model. Lineages refer to those presented in Fig. 1. Statistical values for each lineage are shown (% bootstrap for NJ/% bootstrap MP/% posterior probability for Bayesian). Nodes marked with dash (-) are present in the method but with low statistical support (<50% bootstrap). Asterisks (∗) refer to the sequences obtained from the database.

(see Fig. 1). The species of lineage A, *Leptocottus arma-tus* and out-group spawn in seawater habitats, the remaining freshwater cottoids spawning in freshwater habitats, regardless of life-history type (see Fig. 2a), suggesting that the various life-history types in lineages B–E have evolved from an ancestor which had acquired freshwater tolerance in gametes and embryos.

In lineage D, the fluvial sculpin, *C. pollux* large-egg type, had a sister relationship with the amphidromous sculpin, *C. pollux* middle-egg type. Moreover, in lineage C, such amphidromous–fluvial species' pairs were found independently in different lineages, viz. *C. amblystomop-sis–C. nozawae* pair and *C. hangiongensis–C. koreanus* pair (see Fig. 2b). Amphidromous species in eastern Eurasia are known to spawn a great number of small-sized

eggs, which produce pelagic larvae (Goto, 1981, 1990; Kurawaka, 1976; Shimizu et al., 1994). In contrast, fluvial species deposit a small number of large-sized eggs, from which well-developed benthic juveniles emerge (Byeon et al., 1995; Goto, 1990; Kurawaka, 1976), the former being considered an adaptation to the lower stream environments, and the latter, an adaptation to upstream habitats (Goto, 1990). Such alternations of reproductive strategy and early ontogeny have resulted in evolutionary changes in life-history and subsequent speciation. These parallel situations in lineages C and D suggest that life-history divergence between *Cottus* species (amphidromous–fluvial species' pairs) has been a general adaptive pattern for various freshwater habitats in Japan and adjacent regions.

According to comparisons of early life-history and ontogeny among *Cottus* species, it has been postulated that a fluvial life-history would have been derived from an amphidromous style by way of adaptation to an upstream habitat, involving increased egg size and advanced ametamorphic ontogeny (Goto, 1990; Mizuno, 1963). However, the present study indicated otherwise. Mapping of life-histories onto the ML tree revealed that an amphidromous life-history would have been derived from a fluvial type in the lineages C, D, and E (Fig. 2b). In some aquatic taxa, transitions from diadromous to freshwater life-histories are known to have occurred repeatedly by way of adaptation to freshwater environment (e.g., Gross, 1987; McDowall, 1997). Parallel evolution of adaptive traits may confuse parsimonious reconstructions of the life-histories. In the galaxiid fishes (Galaxiidae), strict parsimonious reconstruction of life-histories based on the molecular phylogenetic tree would favor multiple gains, rather than multiple losses, of diadromy because the former hypothesis requires fewer evolutionary steps (Waters and Wallis, 2001). However, it is considered that the galaxiids represent a number of independent radiations from a migratory stock with repeated loss of diadromy (Waters and Wallis, 2001), because a diadromous life-history is suggested to be primitive in the Galaxiidae fishes (McDowall, 1993). This might also be the case in the freshwater sculpins. In this study, we could not determine confidently the direction of the evolutionary trends of life-history styles. Moreover, spawning habitat and life-history of the common ancestor of lineages B–E could not be reconstructed (equivocal), because of low resolution of interrelationships among lineages. To make it clear, it is indispensable to resolve basal phylogenetic relationships in freshwater cottoids. Phylogenetic reconstruction of various physiological characteristics related to life-history, such as freshwater or seawater tolerance of gametes and larvae, larval developmental strategies, and egg sizes, may lead to a better understanding of the evolutionary trends of life-history styles. More studies are needed on these characters. Nevertheless, the phylogenetic trends in *Cottus* and its relatives, which can produce pelagic and/or benthic larvae, may reflect life-history evolution and speciation, especially in eastern Eurasia, during adaptation to various freshwater habitats.

4.3. Estimation of divergence time

Rate constancy could be assumed in 12S + CR divergence among OTUs, excluding *T. fasciatus*, *C. kazika*, *C. aleuticus*, *C. gobio*, and *C. poecilopus* from Poland, *C. czerskii*, and *C. reinii* sequences. Kontula et al. (2003) estimated ages of 1.2–3.1 million years (Myr) for the root of the monophyletic Baikalian cottoids, using a synonymous rate estimate of cytochrome *b* and ATPase 6.

These estimated divergence times are concordant with other studies (2–2.5 Myr, Kiril'chik et al., 1995; Kiril'chik and Slobodyanyuk, 1997; Slobodyanyuk et al., 1995). Taking the time estimates as appropriate, we obtained from a "linearized tree" for the Baikalian and other cottoids (Fig. 3 in Kontula et al., 2003), 2.5–6.2 Myr as the divergence time among the Baikalian, Siberian, and North American cottoids corresponding to the node for *C. cognatus* and *Leocottus kesslerii* (see Fig. 3). Applying this age estimate to the molecular clock-enforced ML tree, using the 12S + CR dataset, a rate of 1.0–2.4% per Myr was obtained. Subsequently, these rates were used for age estimation, indicating slow (1.0% per Myr) and fast (2.4% per Myr) alternatives.

The root divergence of lineages B–E was estimated as 3.3 Myr (fast rate) or 8.0 Myr (slow rate), suggesting a Pliocene (or late Miocene) radiation of freshwater cottoids, including the ancestor of the Baikalian group. However, it is difficult to estimate the divergence time between lineage A and the lineages B–E, because of the rate heterogeneity observed in lineage A.

4.4. Evolutionary history

Based on the mtDNA phylogeny and estimation of divergence times, the evolutionary process of the freshwater cottoids, included in *Cottus* and *Trachidermus*, including the endemic Baikalian cottoids, was considered as follows. The common ancestor of freshwater sculpins (*Cottus* + *Trachidermus*) can be hypothesized as having been a euryhaline species, similar to *Leptocottus* species. From such an ancestor, lineage A diverged through invasion into freshwater habitats in eastern Asia, all of the species in that lineage having a catadromous life-history, spending most of their life in freshwater habitats and migrating to marine habitats for breeding (Kinoshita et al., 1999; Takita and Chikamoto, 1994). In other words, *T. fasciatus* and *C. kazika* (lineage A) did not acquire freshwater tolerance of gametes and larvae, although adults had adapted to freshwater habitats, suggesting that they have retained ancestral characteristics for breeding.

During the invasion of the above lineage into freshwater habitats, a lineage that had successfully acquired freshwater tolerance of gametes and embryos (the common ancestor of lineages B–E) arose. Gaining the ability to breed in freshwater habitats would have allowed the lineages B–E to invade still further into freshwater habitats. This has resulted in the derivation of almost all the present-day freshwater cottoid species from the lineages B–E. In these lineages, speciation and life-history evolution have occurred in the course of extensive dispersal and adaptation to freshwater habitats from the Pliocene or late Miocene (alternative clock).

Species belonging to lineage E are widely distributed in Europe, Siberia, and North America, suggesting that

they have diversified in each region from an ancestral stock. Since *C. cognatus*, *C. aleuticus*, and *C. bairdii* were suggested to be included in a single lineage (see Figs. 1 and 4), it is possible that the North American *Cottus* species originated from the ancestral stock which invaded the area during the Pliocene (or late Miocene). It is suggested that the Baikalian cottoids would be an extremely specialized group derived from the ancestral stock of lineage E. Present-day cottoid diversity in Lake Baikal is thought to have originated from the single lineage which invaded the ancient lake through adaptive radiation during the late Pliocene–Pleistocene (see Fig. 3; Kontula et al., 2003). Further analysis using much informative markers is needed to examine the hypothesis.

Lineage B consisted of intraspecific variants of *C. poecilopus*, which is widely distributed from Far East Russia to Europe (Berg, 1949; Reshetnikov et al., 1997). HKY + G + I distances for 12S + CR between the Poland and Amur basin samples, which can be regarded as the westernmost and easternmost populations, respectively, were 0.030–0.037, indicating that the phylogeographic pattern of this species may be highly divergent. Because *C. poecilopus* is considered to be a transbasin species in northern Eurasia, as are *Thymallus thymallus*, *Phoxinus phoxinus*, and *Noemacheilus barbatulus toni* (Berg, 1949; Froufe et al., 2003), their comparative phylogeography should provide an insight into the dispersal and formation of the northern Eurasian ichthyofauna.

In eastern Eurasia, one of the most species rich areas, three pairs of fluvial and amphidromous species were found in different lineages (C and D) (see Figs. 1 and 2). Since the *C. amblystomopsis–C. nozawae* pair is distributed in Japan and Sakhalin, but absent in Siberia (Goto, 1980; Goto et al., 2001), their speciation is thought to have occurred in the Japan–Sakhalin region around 0.7 Myr ago (fast rate) or 1.6 Myr ago (slow rate). A recently described sculpin, *C. koreanus* is endemic to the Korean Peninsula (Fujii et al., 2005). Speciation between *C. hangiongensis* and *C. koreanus* might have occurred on the Asian continent around 1.7 Myr ago (fast rate) or 4.0 Myr (slow rate). The *C. pollux* complex, including *C. reinii*, is an indigenous species' group in the Japanese Archipelago (Goto, 2001a; Nakabo, 2002), suggesting that speciation events also occurred in paleo-Japan around 0.4–1.7 Myr ago (fast rate) or 1.0–4.0 Myr ago (slow rate). Such speciation, with alternation of life-history styles, occurred independently in each lineage on the eastern slope of the Eurasian continent and paleo-Japanese Archipelago, suggesting the existence of similar freshwater environmental conditions in both regions during the late Pliocene–Pleistocene (fast clock) or Pliocene–early Pleistocene (slow clock).

Molecular phylogenetic studies of other coldwater-adapted fishes in eastern Asia, such as ninespine sticklebacks (genus *Pungitius*), threespine sticklebacks (*Gasterosteus aculeatus*), and a Japanese fluvial lamprey (genus *Lethenteron*), show that speciation in these fishes occurred in eastern Asia during the Pliocene–Pleistocene (0.8–3 Myr ago) (Higuchi and Goto, 1996; Takahashi and Goto, 2001; Yamazaki and Goto, 1998), as we have shown in *Cottus*. Global climate fluctuations during the Pliocene–Pleistocene influenced freshwater environments in this area (Hewitt, 2000; Lindberg, 1972). The formation of the paleo-Japanese Archipelago and paleo-Sea-of-Japan, supposed to have been a semiclosed cool sea during the Pliocene, are thought to have also had great effect on the freshwater fish habitats at that time (Lindberg, 1972; Nishimura, 1980). Consequently, numerous freshwater fish speciation events in this area might have occurred. In *Cottus* and *Trachidermus* fishes, the eastern Eurasia including Japan might be the place where three major lineages (Lineages A, C, and D) have originated, and is thought to have been a center of their speciation events as suggested by Sideleva (1994).

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