TITLE:

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CITATION:

Minamoto, Toshifumi ...[et al]. Phylogenetic Relationships among Osmerid and Salangid Fish Inferred from Mitochondrial Cytochrome b Gene Sequences. Memoirs of the Faculty of Science, Kyoto University. Series of biology. New series 2002, 18(1): 1-13

ISSUE DATE:

2002-12

URL:

http://hdl.handle.net/2433/258943

RIGHT:

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Mem. Fac. Sci. Kyoto Univ. (Ser. Biol,), 18: 1-13, Dec., 2002

Phylogenetic Relationships among Osmerid and Salangid Fish Inferred from Mitochondrial Cytochrome b Gene Sequences

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(Received November 21, 2002)

Abstract The phylogenetic or taxonomic relationships among Northern Hemisphere osmeroid fish including the families Osmeridae and Salangidae have been investigated mainly on the basis of morphological characteristics, but conclusions from these studies differ markedly and have been controversial. The authors reconstructed molecular phylogenetic trees, based on a 402-base pair region of the mitochondrial cytochrome b gene, among six Osmeridae species from (*Plecoglossus altivelis*, *Hypomesus japonicus*, *Hypomesus transpacificus*, *Osmerus dentex*, *Spirinchus lanceolatus* and *Mallotus villosus*), one Salangidae species (*Salangichthys microdon*), and one Galaxiidae species (*Galaxias maculatus*), a Southern Hemisphere osmeroid. The molecular phylogenetic trees constructed by the maximum parsimony and maximum likelihood methods indicated monophyly of Northern Hemisphere osmeroids, and a sister-group relationship between Galaxiidae and the other species examined. In the Northern Hemisphere osmeroids, the present results suggested that *Plecoglossus* is an ancestral genus, and that there are close relationships between the genera *Osmerus* and *Spirinchus* and between *Salangichthys* and *Mallotus*.

Key words Osmeridae, Salangidae, Molecular phylogeny, Molecular clock

Introduction

Osmerid fish, the smelts and their relatives, show anadoromous, amphidromous, marine and freshwater lifestyles and are widely distributed in the Arctic, Atlantic and Pacific Oceans in the Northern Hemisphere. They show some common morphological characteristics as follows: dumbbell shaped palatine bone, notch in dorsal margin of opercle, absence of pelvic axillary process and presence of adipose fin.

The phylogenetic relationships among them have been discussed by many authors since early times (Chapman 1941; Gosline 1960). Nelson (1994) reported that the family Osmeridae was monophyletic with the inclusion of *Plecoglossus altivelis* Temminck & Schlegel, which was formerly classified as belonging to its own family, Plecoglossidae (Chapman 1941). Phylogenetic relationships among the genera within Osmeridae including *Plecoglossus* were investigated using morphological characters. Howes & Sanford (1987) concluded that *Plecoglossus* was most closely related to *Osmerus*, to which *Hypomesus* formed a sister group, and that *Spirinchus* and *Mallotus* were ancestral osmerids (Fig. 1a). On the other hand, Wilson & Williams (1991) maintained that *Osmerus*, *Spirinchus* and *Plecoglossus* were closely related to one another, and that *Hypomesus* and *Mallotus* had separated from the other osmerids at an early stage (Fig. 1b). In a cladistic

study based on 84 morphological characters, Begle (1991) concluded that *Plecoglossus* and *Osmerus* formed a sister group and suggested that there were close relationships between *Spirinchus*, *Mallotus* and *Hypomesus* (Fig. 1c). Johnson & Patterson (1996) also examined the relationships among osmeroids based on 112 morphological characters using the cladistic method, and concluded that *Hypomesus* and *Plecoglossus* had separated from other osmerids first and second, respectively. They also concluded that *Osmerus* and *Spirinchus* were closely related (Fig. 1d).

Salangids, which are known as icefish or noodlefish, have slender and transparent or translucent bodies, and inhabit the seacoast, rivers and lakes of East Asia. Because of their unique morphological specializations, their phylogenetic positions have been discussed by many authors. Gosline (1960) and Roberts (1984) speculated that salangids had close relations with Osmeridae including *Plecoglossus*. However, the question of whether salangids are nested within Osmeridae or not has yet to be resolved. Howes & Sanford (1987) reported a synapomorphy indicating that the Salangidae were the sister group of Osmeridae (Fig. 1a). Begle (1991) maintained that salangids were not nested in osmerids but were highly modified members of the Southern Hemisphere osmeroids (Fig 1c): including family Retropinnidae and Galaxiidae (Johnson & Patterson 1996). In contrast, Johnson & Patterson (1996) concluded based on cladistic analysis of morphological characters that salangids were nested within osmerids as the sister group of *Mallotus* (Fig. 1d). Thus, the phylogeny of osmerids and salangids based on the morphological studies has been much controversial (Fig. 1).

Mitochondrial DNA (mtDNA) sequence analysis has found widespread use as a tool for phylogenetic studies (Meyer 1993; Miya & Nishida 1996). This method resolved many problems that were not concluded by the morphological characteristics (Horai *et al.* 1995). The fast rate of evolution of mtDNA compared to nuclear DNA makes mtDNA useful for high-resolution analyses in recent branching events. Studies of intra- and interspecies diversity of mtDNA in Salmonidae fish were carried out and the phylogenetic relationships within a genus or among phenotypic forms have been discussed. For example, Thomas & Beckenbach (1989) examined variation in salmonid mtDNA on the basis of sequences from six Pacific salmonid fishes, and reported close relationships among these fishes. Mitochondrial DNA sequences have also been used to analyze the species complex within the genus *Osmerus*. Taylor & Dodson (1994) reported the mtDNA diversification of three smelts belonging to the genus *Osmerus* on the basis of the divergence of a 300-base pair region of cytochrome b gene. More recently, Waters & Burridge (1999) examined the intraspecific mtDNA sequence divergence of *Galaxias maculatus* Jenyns, a representative Southern Hemisphere osmeroid, using cytochrome b and 16S rRNA genes. However, there have been no reports at the molecular level to determine the phylogenetic relationships among the families Osmeridae and Salangidae.

The aim of this study was to investigate the phylogenetic relationships using a 402-base pair sequence of the mitochondrial cytochrome b gene among six osmerid species (*P. altivelis*, *Osmerus dentex* Steindachner, *Hypomesus japonicus* Brevoort, *Hypomesus transpacificus* McAllister, *Spirinchus lanceolatus* Hikita and *Mallotus villosus* Müller), living near Japan, one salangid (*Salangichthys microdon* Bleeker), one galaxiid repre-

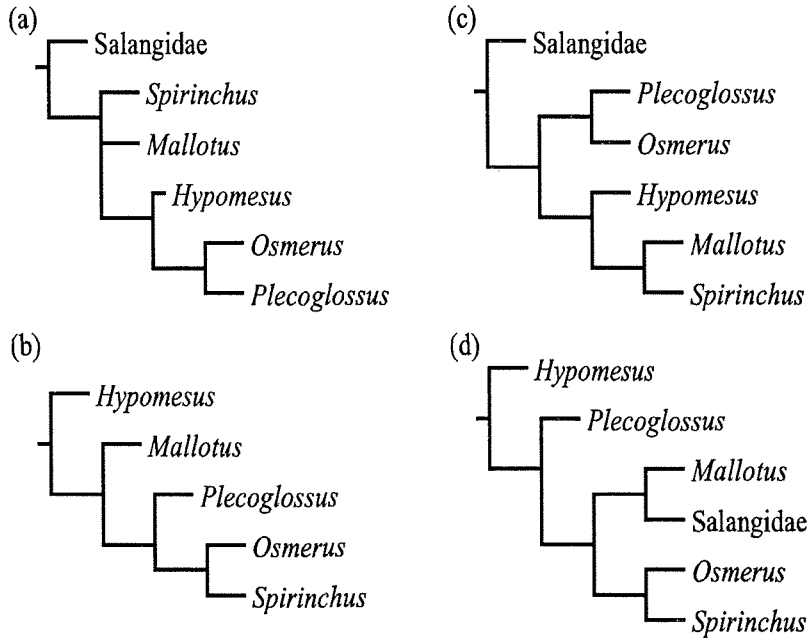

Fig. 1. Phylogenetic relationships among five osmerid genera and Salangidae as proposed by (a) Howes & Sanford (1987), (b) Wilson & Williams (1991), (c) Begle (1991) and (d) Johnson & Patterson (1996). These trees were constructed on the basis of morphological characters.

senting Southern Hemisphere osmeroids (*G. maculatus*) and two salmonids (*Oncorhynchus keta* Walbaum and *Salmo salar* L.) as outgroups, and to compare with the phylogenetic hypotheses proposed by many authors on the basis of morphological characters.

Materials and Methods

Samples

Landlocked forms of *P. altivelis* were collected from Lake Biwa (Shiga Pref., Japan) and amphidromous forms were collected from Iwaki River (Aomori Pref., Japan) and from Korea (Kang-won-do Pref. and Cho'llanamdo Pref.). *H. transpacificus* were collected from Lake Biwa. *H. japonicus* (detailed locality unknown), *O. dentex* collected from Hokkaido Pref., *S. lanceolatus* collected from Mukawa River (Hokkaido Pref., Japan), *M. villosus* (detailed locality unknown), *S. microdon* collected from the Sea of Japan, and *O. keta* collected from Chitose River (Hokkaido Pref. Japan), were purchased from fishermen or fish dealers. All fishes were preserved at -80Åé until DNA extraction.

DNA Sequencing

Total DNA was extracted from frozen muscles of fishes according to the standard method. DNA fragments were amplified using the polymerase chain reaction (PCR) with

the primers L14724 (Meyer *et al.* 1990), 5'-GATATGAAAAACCATCGTTG-3', and H15149 (Kocher *et al.* 1989), 5'-CTCAGAATGATATTTGTCCTCA-3'. We also used the primer H15152, 5'-GCCCCTCAGAATGATATTTGTCC-3' for *M. villosus*. PCR was performed for 25-35 cycles using a DNA thermal cycler model 9700 (Applied Biosystems, CA, USA). The conditions of PCR were as follows: denaturation, 94℃ for 1 min; annealing, 45℃ for 1 min; and extension, 72℃ for 1.5 min. PCR products were purified using Gene Clean II (BIO 101, Inc., CA, USA) after separation by agarose electrophoresis and used as templates for direct sequencing. Sequencing reactions were carried out by the dye terminator cycle sequencing method according to the manufacturer's protocol (Applied Biosystems). All sequences were determined in both directions with an ABI 373S automatic DNA sequencer (Applied Biosystems).

All DNA sequences obtained in this study were submitted to GenBank. Accession numbers are AB049012-AB049018 (*P. altivelis*), AB089609 (*H. transpacificus*), AB049019 (*H. japonicus*), AB049020 (*O. dentex*), AB049021 (*S. lanceolatus*), AB049022 (*M. villosus*), AB049023 (*S. microdon*) and AB049024 (*O. keta*). In the molecular phylogenetic analysis, landlocked type of *P. altivelis* (AB049016) was employed. We obtained the sequences of *S. salar* (AF165083) and *G. maculatus* (AF007023) from GenBank.

Phylogenetic analysis

The CLUSTAL W (Thompson *et al.* 1994) computer software was used to align the 402-base pair sequences. Maximum parsimony (MP) tree and maximum likelihood (ML) tree were constructed using PAUP* 4.0b10 computer software (Swofford 2000). In constructing these trees, two salmonids (*O. keta* and *S. salar*) were used as outgroups. MP analysis, in which nucleotide sites were equally weighted, and character state transformations were treated as unordered and of equal cost, was performed using the exhaustive algorithm. The ML tree was constructed using the exhaustive algorithm. In this analysis, transition / transversion ratio was set to 2, and the Hasegawa-Kishino-Yano model (Hasagawa *et al.* 1985) was used as the base substitution model. To evaluate the robustness of the internal branches, bootstrapping percentages (1000 replications) were also calculated using the Heuristic algorithm in PAUP* 4.0b10.

Results

Mitochondrial DNA sequence variations

First, the 402-base pair region of the mitochondrial cytochrome b gene from 12 amphidromous *P. altivelis* (4 from Japan and 8 from Korea), and nine landlocked fish collected from Lake Biwa were sequenced. Five positions (51, 330, 363, 393 and 402) of 402 sites were found to be variant. We could find no form-specific substitutions in these mitochondrial cytochrome b sequences between amphidromous and landlocked fishes (Iguchi *et al.* 1997). Of the 12 amphidromous fishes, five had 'GAAC(A/C/G)', four had 'AAAC(A/G)', two had 'AGACG' and one had 'GAGTG'. All nine landlocked fishes had

'AAAC(A/G)' at these five positions: landlocked fishes showed lower variation than amphidromous fishes as reported by Iguchi *et al.* (1997). We used the sequence of the landlocked type of *P. altivelis* ('AAACA' at position 51, 330, 363, 393 and 402), which had one substitution with mtDNA genome sequence reported previously (Ishiguro *et al.* 2001), in the following molecular phylogenetic analysis: the results were not different when the sequences of the amphidromous fishes were employed.

The first 402-base pair regions of the mitochondrial cytochrome b gene from five osmerids including landlocked type of *P. altivelis*, one salangid and one salmonid were sequenced. Taylor & Dodson (1994) reported 300-base pair sequences of *O. dentex* and *M. villosus*. We compared our sequences of these two fishes with the corresponding region (300-base pair) reported by Taylor & Dodson (1994), and found that only two and three bases were different in *O. dentex* and *M. villosus*, respectively.

The substitution sites of the aligned sequences for the species examined were shown in Table 1. Base frequencies of the sequences from nine species were 0.23 ± 0.02 (A), 0.30 ± 0.01 (C), 0.17 ± 0.01 (G) and 0.31 ± 0.01 (T) (Mean ± S.D.). All sequences were biased against guanine on the light strand, indicating that these sequences were not those of a paralogous gene but were indeed those of authentic mtDNA (Zhang & Hewitt 1996). Scatter plots, which show the ratio of transitions in total substitution to the ratio of total sequence divergence, did not show a transition plateau (Fig. 2), indicating that our data were useful for phylogenetic inference (Brown *et al.* 1982).

Sequence divergence derived from 402-base pair region of cytochrome b and the number of nucleotide differences observed are shown in Table 2. The sequence diver-

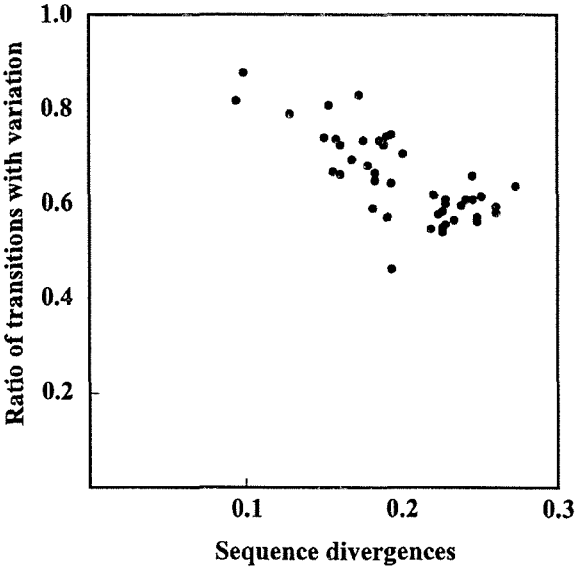

Fig. 2. Scatter plots of each sequence pair examined. The horizontal axis is the ratio of sequence divergence, and the vertical axis is the ratio of transitions with the variation.

Table 1. Positions of the nucleotide substitution in the partial portion of cytochrome b gene (402 bp)

Species	8	9	10	12	15	18	21	24	27	28	30	31	33	39	40	41	42	45	47	51	52	54	57	60	61	63	66	69	72	75	77	78	81	84	85	87	90	96	99	102	105	108	109	111	114	115	117	120	121	123	124	125	126	128
P. altivelis	A	C	C	T	G	G	C	C	C	C	C	T	A	T	A	G	T	C	A	A	C	C	C	C	C	G	C	C	C	A	A	C	T	G	A	C	G	C	T	C	T	T	C	T	G	C	T	C	C	T	A	T	C	T
H. japonicus	G	.	.	C	C	A	.	T	.	.	.	C	T	.	.	C	C	.	.	C	.	A	T	.	.	.	.	.	.	C	.	T	.	A	.	.	A	.	.	.	A	C	.	C	T	T	A	T	.	.	.	.	.	.
H. transpacificus	.	.	.	.	.	A	.	.	.	.	.	C	.	.	.	C	C	T	.	C	.	A	T	T	T	A	T	A	.	C	.	T	.	A	.	.	A	.	.	A	C	.	.	.	.	.	G	T	.	.	.	.	T	.
O. dentex	.	.	.	.	.	A	.	.	.	.	.	C	.	.	.	C	C	T	.	C	.	A	T	T	.	A	T	G	.	C	.	T	.	T	G	G	A	.	C	.	C	.	.	.	.	.	C	T	.	C	.	.	T	.
S. lanceolatus	.	.	.	.	.	A	.	.	.	A	.	C	.	.	.	C	C	T	.	C	.	A	T	T	.	A	T	A	.	T	.	T	.	C	G	.	A	.	C	.	C	C	.	.	.	.	C	T	.	.	.	.	.	.
M. villosus	G	.	.	.	.	A	.	.	.	.	.	C	G	.	.	C	C	T	.	C	.	A	T	T	.	C	T	A	.	C	.	.	C	C	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	C	.	.	T	.
S. microdon	.	.	A	.	A	A	.	.	.	.	.	C	G	.	.	C	.	T	.	T	T	A	T	.	T	A	T	G	.	G	G	.	.	A	.	T	.	T	.	.	C	A	.	.	.	T	G	.	.	.	.	.	T	.
G. maculatus	.	T	.	A	A	.	.	.	.	.	A	C	.	C	G	C	C	.	G	G	.	T	T	.	.	.	A	.	.	.	.	.	C	A	G	.	A	.	C	A	.	C	.	.	C	.	.	.	T	G	G	C	T	G
O. keta	.	.	.	C	A	A	.	T	T	.	.	C	.	C	G	C	C	T	.	.	.	A	.	.	.	C	A	A	A	T	.	.	C	A	G	.	A	.	.	T	A	C	.	G	C	T	A	.	.	A	G	C	.	C
S. salar	.	.	.	C	A	A	T	.	G	.	.	C	.	.	G	C	.	T	.	.	.	A	.	T	.	C	A	A	A	T	.	.	C	A	G	T	A	.	.	.	A	C	T	A	C	.	A	T	.	A	G	C	.	C

Species	129	132	135	138	141	144	147	150	151	153	156	159	162	165	168	169	171	174	176	177	178	180	183	186	189	192	195	198	199	200	201	205	207	210	213	216	219	221	222	225	228	232	234	237	240	243	246	249	252	258	264	270	273	276
P. altivelis	C	G	T	T	C	C	A	T	T	A	C	G	C	C	A	G	T	G	C	C	G	C	G	G	C	C	A	T	G	T	C	C	C	C	T	C	C	A	C	C	C	T	A	C	G	C	G	T	T	A	C	C	C	C
H. japonicus	.	A	C	A	A	.	.	.	.	.	.	.	.	T	T	.	C	.	.	T	.	T	A	A	T	T	.	C	.	.	G	.	T	.	A	.	.	.	.	.	.	C	.	.	.	.	.	C	.	.	T	.	T	.
H. transpacificus	.	A	C	.	G	.	C	.	.	G	T	.	.	T	T	.	.	.	.	T	.	T	C	T	T	.	T	.	.	.	T	.	.	.	A	.	T	.	T	.	.	C	.	.	T	.	.	C	.	.	T	.	T	T
O. dentex	T	.	C	C	A	.	.	C	C	G	.	.	.	.	T	.	C	.	.	T	.	.	A	.	.	.	C	C	.	.	A	.	.	.	G	T	.	.	.	.	T	C	.	T	.	.	.	C	.	.	.	.	T	.
S. lanceolatus	.	A	C	.	G	.	.	.	C	.	.	.	.	.	T	.	C	A	.	T	.	.	A	.	T	.	T	.	.	.	T	.	.	.	G	.	.	.	T	.	T	C	.	.	A	T	.	C	.	G	T	.	.	.
M. villosus	T	.	.	.	A	.	.	.	C	G	.	A	T	.	T	.	C	.	.	T	.	.	A	A	.	.	C	A	.	.	A	.	A	.	.	.	A	.	T	T	.	A	.	.	A	.	.	.	.	.	.	.	T	T
S. microdon	.	A	C	C	A	.	C	C	C	C	A	A	.	.	C	.	G	.	.	.	T	.	C	A	.	.	T	A	.	.	T	.	A	.	G	.	T	.	.	.	.	C	C	.	A	T	.	.	C	.	T	T	.	T
G. maculatus	.	.	C	.	G	A	T	.	C	T	T	A	.	T	T	T	.	T	T	.	T	T	T	A	T	T	.	C	A	C	A	A	.	T	A	T	A	G	.	.	.	.	G	.	.	.	.	C	.	.	T	T	.	.
O. keta	.	A	.	.	.	G	C	C	C	.	.	.	.	.	C	T	C	C	T	T	T	A	A	T	T	.	T	C	T	G	.	A	.	.	A	T	.	G	.	.	.	C	.	T	.	.	C	C	.	.	T	T	T	T
S. salar	.	A	C	.	.	G	C	C	C	.	.	A	.	.	C	T	C	T	T	.	T	A	A	T	T	.	T	.	T	G	.	A	T	.	A	T	T	G	.	T	.	C	C	.	T	.	T	C	.	.	T	.	T	.

Species	279	282	285	286	288	291	294	296	297	300	303	304	306	309	312	315	318	320	321	324	327	330	333	336	339	341	342	343	345	348	351	352	354	357	358	360	363	364	366	367	368	369	372	375	378	380	381	384	387	390	393	396	399	402
P. altivelis	T	C	C	C	T	C	T	G	T	A	A	C	T	C	T	G	T	T	C	T	C	A	G	A	A	A	C	A	C	T	T	G	C	T	C	C	A	C	A	G	T	T	G	A	T	C	T	T	A	C	C	C	T	A
H. japonicus	.	T	T	.	.	T	.	.	.	.	T	.	.	.	.	C	.	.	.	C	.	G	.	C	.	.	T	.	.	.	G	.	.	C	.	T	C	T	G	.	.	.	A	G	.	.	.	.	T	.	T	A	.	T
H. transpacificus	C	T	.	.	.	T	.	.	C	.	T	.	.	T	C	C	A	.	.	G	.	G	A	T	.	.	.	.	.	.	G	.	T	A	.	A	T	T	.	.	.	C	A	G	C	.	.	.	G	.	T	T	.	C
O. dentex	.	T	.	.	.	T	.	.	C	G	T	.	.	.	C	C	.	.	.	.	.	G	A	C	.	.	.	.	T	C	A	.	.	.	.	.	C	T	.	.	.	.	.	.	.	.	C	C	.	.	T	.	C	C
S. lanceolatus	C	T	T	.	.	T	.	.	C	.	T	.	.	.	C	C	.	.	.	.	.	.	A	C	.	.	.	G	G	C	A	.	.	.	.	.	T	T	.	.	.	G	.	.	A	G	C	C	.	.	T	.	C	T
M. villosus	.	T	.	.	C	T	C	.	C	.	G	.	.	.	C	C	.	.	.	.	T	.	.	T	.	C	.	G	.	C	G	.	.	.	.	.	G	.	.	.	.	.	.	G	.	.	C	.	.	.	T	A	C	T
S. microdon	C	.	.	.	C	T	.	.	C	C	C	.	C	.	C	T	C	.	.	C	.	G	.	T	.	.	.	G	T	.	C	.	.	C	.	T	C	.	.	.	.	G	.	.	.	.	.	.	G	.	.	A	C	T
G. maculatus	.	T	T	A	G	.	C	.	G	.	T	T	G	.	.	C	.	A	T	G	.	.	.	C	.	C	.	.	.	A	A	A	T	C	.	.	C	.	T	.	.	C	.	G	C	.	.	.	T	A	.	T	C	C
O. keta	.	T	T	A	A	T	C	C	C	G	.	.	.	T	C	A	C	A	.	G	.	.	A	C	G	.	T	.	.	A	.	.	A	.	T	A	T	.	C	A	C	.	A	.	.	.	A	C	G	.	.	.	C	G
S. salar	.	T	T	A	A	.	C	C	C	.	.	.	.	T	.	T	C	A	T	A	T	.	A	C	.	.	T	.	.	A	.	.	A	.	.	A	T	.	C	A	C	.	A	.	.	.	C	C	.	.	.	T	.	.

Dots indicate the identical nucleotides with those of the first line (*P. altivelis*).

Table 2. Sequence divergence derived from 402-base pairs of the cytochrome b gene (upper portion of matrix) and the number of nucleotide differences (lower portion of matrix).

Taxon	1	2	3	4	5	6	7	8	9	10
1 *P. altivelis*		0.164	0.182	0.162	0.169	0.157	0.194	0.226	0.241	0.234
2 *H. japonicus*	66		0.137	0.152	0.157	0.174	0.199	0.221	0.219	0.229
3 *H. transpacificus*	73	55		0.142	0.129	0.162	0.174	0.232	0.229	0.229
4 *O. dentex*	65	61	57		0.095	0.129	0.187	0.246	0.224	0.239
5 *S. lanceolatus*	68	63	52	38		0.152	0.189	0.251	0.226	0.226
6 *M.villosus*	63	70	65	52	61		0.179	0.246	0.249	0.249
7 *S. microdon*	78	80	70	75	76	72		0.274	0.261	0.261
8 *G.maculatus*	91	89	93	99	101	99	110		0.229	0.229
9 *O. keta*	97	88	92	90	91	100	105	92		0.100
10 *S.salar*	94	92	92	96	91	100	105	92	40	

gence between galaxiid and salangid, between galaxiid and osmerids, and between salangid and osmerids was 27.4%, 22.1-25.1% and 17.9-20.1%, respectively.

Phylogenetic relationships

We constructed MP and ML trees from the first 402-base pair region of cytochrome b gene of the osmeroid fish using salmonid fish as outgroups (Fig. 3). The topologies of MP and ML tree were identical, and both trees showed a monophyletic relationship among Northern Hemisphere osmeroids (Osmeridae and Salangidae), a sister relationship between these fish and galaxiid, representing Southern Hemisphere osmeroid, and non-monophyletic relationships of two *Hypomesus* species. The bootstrapping percentage of the node from which galaxiids branched from Northern Hemisphere osmeroids was high in both trees. The statistical values of the MP tree were as follows: tree length = 410, consistency index = 0.60, and retention index = 0.45. In ML analysis, likelihood was calculated by the Hasegawa-Kishino-Yano model (Hasegawa *et al.* 1985), and -Ln likelihood was 2309.65.

Molecular phylogenetic trees clustered *Osmerus* and *Spirinchus*, and also clustered *Mallotus* and *Salangichthys*. Next, *Osmerus-Spirinchus* group and *Mallotus-Salangichthys* group were clustered. In these trees, *Plecoglossus* was located at the basal position of the Northern Hemisphere osmeroids, and this tree showed that two *Hypomesus* species were separated after the separation of *Plecoglossus*.

Discussion

Phylogenetic position of salangids

Our molecular phylogenetic trees inferred from the mitochondrial cytochrome b gene sequences showed that *S. microdon* is nested among osmerid fish as a sister group of *Mallotus* (Fig 3). All modern studies of salangid classification based on morphological observations agree in placing them in the order Salmoniformes, and many authors have

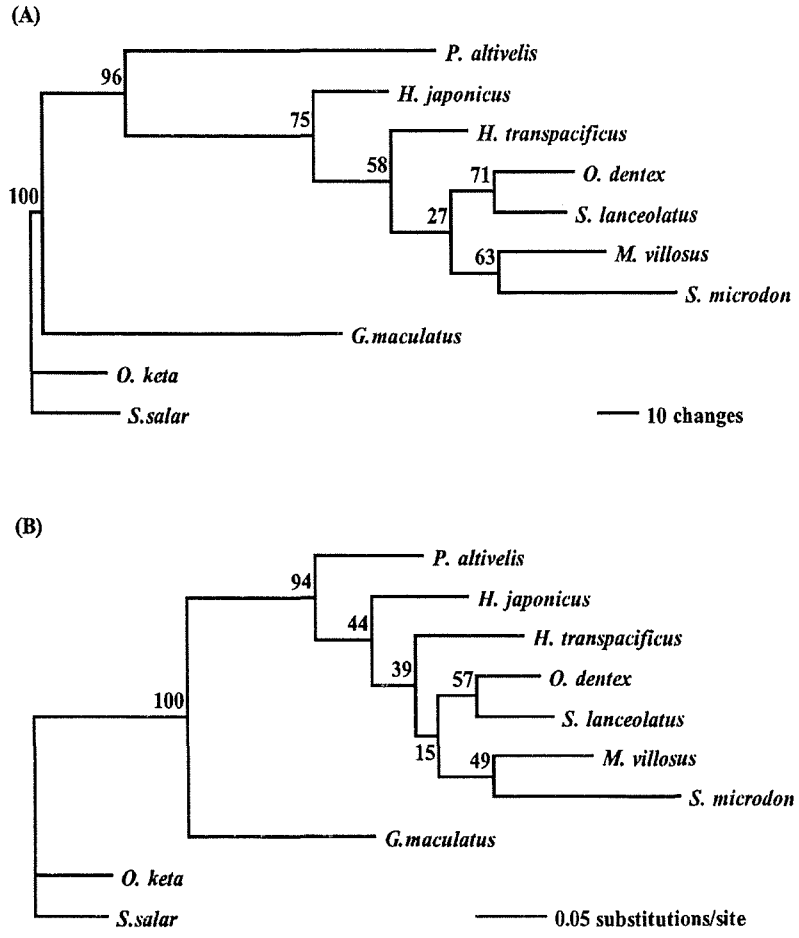

Fig. 3. MP (A) and ML (B) tree among galaxiid, salangid and osmerid fish constructed from sequences of the first 402-base region of the cytochrome b gene. Two salmonid fish (*O. keta* and *S. salar*) were used as outgroups. The scale bars indicate 10 changes in MP tree (A) and 0.05 substitutions/site ML tree (B). Numbers at branching points indicate the bootstrapping probabilities (1000 replications).

associated them with osmeroids (Review in Roberts 1984). However, because of the large number of unique specializations they display, it has not been possible to resolve whether salangids are nested within Osmeridae or not. Howes & Sanford (1987) reported only a single synapomorphy, diabolo-shaped palatine, indicating the monophyly of the family Osmeridae, and a single synapomorphy, the presence of adipose fin cartilage, showing the Salangidae as a sister group of the Osmeridae (Fig. 1a). Begle (1991) showed that there were many shared morphological characters between salangids and Southern Hemisphere osmeroids, for example, posteroventrally curved infraorbital sensory canals, absence of mesocoracoid, and posteriorly positioned dorsal fin. He concluded that they were just highly modified members of the Southern Hemisphere osmeroids on the basis of cladistic analysis, though salangids inhabit in Northern Hemisphere (Fig. 1c). In con-

trast, Johnson & Patterson (1996), who also used cladistic analysis, showed four synapomorphies to *Salangid-Mallotus* taxa, which are long and unossified ethomoids, distally branched fourth pectral radial, fenestrate plated adipose cartilage, and modification of the anal fin skeleton in males. They maintained that some shared characters between salangids and Southern Hemisphere osmeroids were homoplasies and concluded that salangids were nested within osmerids, as a sister group of *Mallotus* (Fig. 1d). Our present result of the molecular phylogenetic study showed that *S. microdon* is well nested in Northern Hemisphere osmeroids (Fig. 3), not clustered with Southern Hemisphere galaxiid, and supports the conclusion of Johnson & Patterson (1996). On the other hand, Saruwatari *et al.* (1999) and Waters *et al.* (2002) reconstructed the molecular phylogenetic tree of lower Euteleosts using partial sequences of mitochondrial DNA sequences and concluded that Salangidae and Osmeridae fish have sister relationship. Further study with more molecular data will be required.

Phylogenetic relationships among Northern Hemisphere osmeroids

What is an ancestral osmerid and what is a derived one is still controversial. The phylogenetic position of *Plecoglossus* has been especially discussed and disputed. *Plecoglossus altivelis* is distributed throughout the Japan Islands, Ryukyu Islands, Taiwan Islands, the Korean Peninsula and some parts of the Chinese Continent (Nishida 1986). It shows some morphological and ecological differences from other osmerids: it grazes adherent algae in streams using comb-like teeth, unlike other osmerids (Kawanabe 1972).

Chapman (1941) concluded that *Plecoglossus* was more closely related to the osmerids than to the other salmonid fish according to many common characters of their bones, general resemblance of the skull, and size and shape of the adult fish. On the other hand, Howes & Sanford (1987) reported that *Plecoglossus* was most closely related to *Osmerus* based on three synapomorphies, which are the presence of symphysial dentary cartilage, a symphysial dentary process and insertion of lavator arcus palatini on the metapterygoid lateral shelf. They also maintained that *Hypomesus* and *Osmerus-Plecoglossus* taxa form a sister-group from a shared character, an ectopterygoid frange (Fig. 1a). Begle (1991) supported the close relationship between *Plecoglossus* and *Osmerus* on the basis of the symphysial process and cartilage, and pterosphenoid interdigitating ventrally with prootic. However, he concluded that *Hypomesus* was not close to *Plecoglossus-Osmerus* taxa (Fig. 1c). In a cladistic study based on 112 morphological characters, Johnson & Patterson (1996) concluded that *Hypomesus* was the most ancestral osmerid according to four primitive characters, which are endopterygoid teeth in a patch posteriorly, the position of the metapterygoid, all epineurals originating on the neural arch, and an unmodified fourth pectral radial. They also concluded that *Plecoglossus* had separated second on the basis of four other primitive characters, presence of vomerine shaft, uninflated otic bulla, parietals fully in contact, and distal keels on the last few neural and haemal arches (Fig. 1d). Molecular phylogenetic tree proposed by Saruwatari *et al.* (1999) and Waters *et al.* (2002) showed that *Plecoglossus* was separated from other osmerids first.

Our molecular phylogenetic trees obtained here inferred from mitochondrial cytochrome b sequences showed that *Plecoglossus* and *Hypomesus* were separated first and

second, respectively, from other Northern Hemisphere osmeroids (Fig. 3). This result supports Johnson & Patterson (1996) who concluded that *Plecoglossus* and *Hypomesus* are the basal osmerids, although the order of branching was not identical.

Another important point of this study was that the molecular trees suggested a close relationship between the genera *Osmerus* and *Spirinchus* (Fig. 3), as has also been discussed by many authors. As mentioned above, Begle (1991) clustered *Plecoglossus* and *Osmerus*, and maintained the monophyly of *Hypomesus*, *Mallotus* and *Spirinchus* on the basis of a narrow levotor process on the fourth epibranchial and narrow a rodlike spine on sphenotic from which the dilator operculi originates (Fig. 1c). However, Wilson & Williams (1991) reported a close relationship between *Osmerus* and *Spirinchus* based on a synapomorphy, a modified anal fin in males (Fig. 1b). Johnson & Patterson (1996) supported their conclusions on the basis of five primitive characters, for example, the absence of cartilaginous interobital septum, and the form of the lower jaw (Fig. 1d). Our molecular phylogenetic trees supported the hypothesis that *Osmerus* and *Spirinchus* are closely related as proposed by Wilson & Williams (1991) and Johnson & Patterson (1996). Further experiments to analyze the sequences of another gene region of related fish including *Allosmerus* and *Thaleichtys* might give us more insight to the phylogenetic relationship of *Osmerus* and *Sprinchus*.

Molecular clock in osmeroid fish

Orti *et al.* (1994) provided an approximate fossil-based calibration for cytochrome b sequence divergence in the family Gasterosteidae of about 2.8% per million years. Taylor & Dodson (1994) applied this rate to estimate the date of separation of *Osmerus* and *Mallotus*. Mitochondrial cytochrome b divergence between Northern Hemisphere osmeroids and galaxiid (22.1-27.4%) and the 2.8% rate of divergence yields a separation date of 7.9-9.8 million years ago (Mya). This calibration also provided a separation date of 7.8-9.3 Mya between osmeroids and salmonids. However, the oldest fossil osmerid is the Paleocene (58-67 Mya) freshwater *Speirsaenigma lindoei*, whose closest living relative is considered to be *P. altivelis* (Wilson & Williams 1991).

We estimated the separation date between osmeroids and salmonids as being in the Paleocene or earlier, and obtained a rate of 0.45% per million years or slower, approximately one-sixth of the rate of 2.8% reported for the Gasterosteidae. Martin *et al.* (1992) and Cantatore *et al.* (1994) reported that the rates of mtDNA evolution in fish are slower than those of mammals with a conventional clock calibration of 2% (Brown *et al.* 1979). By applying the rate determined above (0.45% or slower), we obtained a separation date between Northern and Southern Hemisphere osmeroids of approximately 49 Mya or earlier. It is also suggested that Northern Hemisphere osmeroids might be diversed at the period of 29-45 Mya or earlier.

Ackknowledgements

This work was supported partly by a Grant-in-aid for Scientific Research (09NP1501, 12308028) from the Ministry of Education, Science, Sports and Culture of Japan. The authors are grateful to Dr. K. Iguchi (National Research Institute of Fisheries Sciences, Ueda Branch, Nagano Pref., Japan) who kindly provided samples of amphidromous *P. altivelis*, and to Ms. A. Ishida for her technical assistance.

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