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Evolutionary and taxonomic relationships among Far-Eastern salmonid fishes inferred from mitochondrial DNA divergence

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Mitochondrial DNA (mtDNA) restriction analysis was used to examine the evolutionary and taxonomic relationships among 11 taxa of the subfamily Salmoninae. The genera *Brachymystax* and *Hucho* were closely related, diverging by sequence divergence estimates of 3·1%. Because the mtDNA sequence divergence between blunt- and sharp-snouted forms of *Brachymystax* (2·24%) was similar to divergence level of *Brachymystax* and *Hucho*, then taking into account the distinct morphological, ecological and allozyme differences between them, it is possible to recognize these forms as two separate species. The subgenus *Parahucho* formed a very distinct group differing by 6·35–7·08% (sequence divergence estimate) from both *Brachymystax* and *Hucho* and must be considered as a valid genus. The UPGMA and neighbour-joined phenograms showed that the five genera studied are divided into two main groupings: (1) *Hucho*, *Brachymystax* and *Salvelinus*; and (2) *Oncorhynchus* and *Parahucho* species. The mtDNA sequence divergence estimates between these groupings were about 8·1%. However, the subsequent bootstrap analysis of mtDNA RFLP data did not support the monophyly of the latter grouping. The concordance of morphological and mtDNA phylogenetic patterns is discussed.

Key words: salmonid fishes; mitochondrial DNA; phylogeny.

INTRODUCTION

The taxonomy and evolutionary relationships of Asiatic salmonid genera (*Brachymystax*, *Hucho* and *Parahucho*) to other genera of the subfamily Salmoninae (*Salmo*, *Salvelinus*, *Oncorhynchus*) remains uncertain. Usually, after Tchernavin (1939) and Norden (1961), the genus *Brachymystax* is considered as primitive and its origin is connected with the first round of divergence of the common ancestor of Salmoninae, which further evolution resulted in the successive origin of *Hucho* (including *Parahucho* as subgenus), *Salvelinus*, *Salmo* and *Oncorhynchus* (e.g. Smith & Stearley, 1989; Phillips & Pleyte, 1991). This evolutionary hypothesis is based on morphological data and assumes the primary freshwater origin of Salmoninae. However, recent karyological and biochemical data do not support this hypothesis. The freshwater genera *Brachymystax* and *Hucho* are very similar and their ancestral forms diverged after the phyletic line of anadromous *Parahucho* has arisen (Anbinder *et al.*, 1982; Viktorovsky *et al.*, 1985; Osinov, 1991).

In the present study, the evolutionary and taxonomic relationships among 11 taxa representing five genera of Salmoninae (*Brachymystax*, *Hucho*, *Parahucho*, *Salvelinus* and *Oncorhynchus*) were determined using mitochondrial DNA

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(mtDNA) restriction analysis. Previous analyses of mtDNA variation within the Salmoninae subfamily have been restricted to comparisons of only a few species of two or three genera (e.g. Berg & Ferris, 1984; Gyllensten & Wilson, 1987; Ginatulina *et al.*, 1988; Grewe *et al.*, 1990; McVeigh & Davidson, 1991; Shedlock *et al.*, 1992).

MATERIAL AND METHODS

A total of 168 specimens representing 11 taxa were sampled (Table I). Mitochondrial DNA was purified from liver by the alkaline extraction method (Palva & Palva, 1985). Aliquots of mtDNA were digested with one multihexameric (*Hinc* II) and 15 hexameric (*Bam* HI, *Bgl* I, *Bgl* II, *Cfr* 42I, *Eco* RI, *Eco* 32I, *Eco* 81I, *Eco* 91I, *Eco* 105I, *Hind* III, *Nco* I, *Pvu* II, *Pst* I, *Sca* I and *Xba* I) restriction enzymes in conditions recommended by the supplier (Fermentas, Lithuania). Restriction fragments were separated on 0·6–1·2% agarose gels and visualized by UV radiation after ethidium bromide staining. Fragments were sized by comparison with 1-kilobase ladder standard (Bethesda Research Labs). No attempt to visualize fragments less than 400 base pairs (bp) was made. However, in some cases small fragments were detected by incomplete digestions.

Because of the large number of taxa examined and the high mtDNA diversity observed, it was not feasible to map all restriction sites. Therefore, further analysis was limited to the fragments themselves. The mtDNA fragment data were already utilized for assessing evolutionary relationships among several species of Salmoninae (e.g. Berg & Ferris, 1984; Gyllensten & Wilson, 1987; Ginatulina *et al.*, 1988). The results of these studies were almost completely concordant with those based on restriction site (Thomas *et al.*, 1986) or sequence (Thomas & Beckenbach, 1989; McVeigh & Davidson, 1991; Shedlock *et al.*, 1992) comparisons. Accordingly, we considered it justifiable to estimate evolutionary relationships using restriction fragment analysis in this work.

Distinct single endonuclease patterns were designated by a specific letter in order of appearance. Each fish was assigned a multiletter code which described its composite mtDNA genotype. The composite data were also summarized in a presence/absence matrix of all mtDNA fragments, which was then employed to compute p (the average number of substitutions per nucleotide site) between genotypes according to Nei & Li (1979). The resulting distance matrix was used to construct a phenogram using both constant (UPGMA; Sokal & Sneath, 1963) and varying evolutionary rate [neighbour-joined method (NJ); Saitou & Nei, 1987; tree was routed by midpoint] clustering methods in the NTSYS package (Rohlf, 1993).*

The robustness of specific nodes of the resulting trees was then tested by the bootstrap method (Felsenstein, 1985). Using an original Pascal computer program (written by S.V.S.), we resampled our data (all fragment patterns for each of 16 restriction enzymes here, not individual fragments) to construct 100 fictional sets of data. Each of these was constructed by the iterative sampling of 16 restriction enzymes from an original set of data with replacement. For each fictional set of data we made a presence/absence matrix of all mtDNA fragments, which then was used for estimation of sequence divergence between fictional mtDNA genotypes and following construction of the UPGMA and NJ trees.

RESULTS

Fifteen of the 16 restriction endonucleases used produced polymorphic patterns and resolved 57 fragments per mtDNA genotype on average. A total of 29 composite genotypes was found among the 11 taxa examined (Table II,

*The parsimony methods of reconstruction of phylogeny were not employed because of violation of the requirement of independence characters—the gain or loss of a fragment (character) affects the presence of other fragments produced by a particular restriction enzyme.

TABLE I. List of taxa sampled with their sample size and origin

Species	No. fishes	Origin
Lenok *Brachymystax lenok* (Pallas)		
Blunt-snouted form	43	Various rivers of the Sea of Japan basin, Primorye
	33	Various rivers of the Ussuri (Amur) basin, Primorye
Sharp-snouted form	23	Various rivers of the Ussuri (Amur) basin, Primorye
Taimen *Hucho taimen* (Pallas)	16	Various rivers of the Ussuri (Amur) basin, Primorye
White spotted charr *Salvelinus leucomaenis* (Pallas)	6	The Edinka river of the Sea of Japan basin, Primorye
Dolly varden *Salvelinus malma* (Walbaum)	6	The Edinka river of the Sea of Japan basin, Primorye
Sakhalin taimen *Parahucho perryi* (Brevoort)	5	The Edinka river of the Sea of Japan basin, Primorye
Kamchatka trout *Oncorhynchus mykiss* (Walbaum)	3	The Kamchatka river, Eastern Kamchatka
Coho salmon *Oncorhynchus kisutch* (Walbaum)	2	The Kievka river of the Sea of Japan basin, Primorye
Masu salmon *Oncorhynchus masou* (Brevoort)	12	Various rivers of the Sea of Japan basin, Primorye
Chum salmon *Oncorhynchus keta* (Walbaum)	16	Various rivers of the Sea of Japan basin, Primorye
Pink salmon *Oncorhynchus gorbuscha* (Walbaum)	3	The Edinka river of the Sea of Japan basin, Primorye

TABLE II. Composite clonal genotypes (rows of letters) observed in the assayed salmonid fishes

Species	mtDNA clone	Composite*															
B. lenok																	
Blunt-snouted form	BJ1	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
	BJ2	A	A	B	A	A	B	B	A	A	B	A	A	A	A	B	A
	BJ3	A	A	B	A	A	A	B	A	A	A	A	A	A	A	A	B
	BU1	A	A	A	A	A	A	B	A	A	B	A	A	B	A	C	A
	BU2	A	A	A	A	A	A	B	A	A	A	A	A	B	A	C	A
	BU3	A	B	A	A	A	C	A	B	A	A	B	B	A	A	D	C
Sharp-snouted form	SU1	A	B	A	A	A	C	A	B	A	A	B	B	B	A	D	C
	SU2	A	C	A	A	A	C	A	B	A	A	B	B	B	A	D	C
	SU3	A	B	A	A	A	C	A	B	A	A	C	B	A	A	D	C
	SU4	A	B	A	A	A	C	A	B	A	B	B	B	B	A	D	C
H. taimen	TAI	A	D	A	A	A	D	A	C	A	C	A	C	C	B	E	B
S. leucomaenis	KU1	B	E	C	A	B	E	C	E	B	D	C	D	D	C	F	D
	KU2	B	E	C	A	B	F	C	D	B	D	C	D	D	C	F	D
S. malma	MA1	A	F	D	A	B	G	D	F	C	F	A	D	E	C	G	C
	MA2	A	F	E	A	B	G	D	F	C	F	A	D	E	C	G	C
	MA3	A	F	D	A	B	G	D	F	C	E	A	D	E	C	G	C
P. perryi	SAH	C	G	F	A	A	H	E	G	D	G	D	E	F	B	H	E
O. mykiss	MYK	D	H	G	A	C	I	F	H	E	H	E	F	G	D	I	F
O. kisutch	COH	D	I	H	A	C	K	G	I	F	I	F	G	G	E	K	G
O. masou	MS1	E	K	H	A	E	L	D	H	G	L	G	H	H	A	L	F
	MS2	E	K	I	A	E	L	D	H	G	L	G	H	H	A	L	F
	MS3	E	K	I	A	E	L	D	H	G	K	G	H	H	A	L	F
	MS4	E	K	H	A	E	L	D	H	G	K	G	H	H	A	L	F
O. keta	CH1	F	G	K	A	C	M	H	K	H	M	H	I	I	F	M	I
	CH2	F	G	K	A	C	M	G	K	H	M	H	I	I	F	M	I
	CH3	F	G	K	A	D	M	G	K	H	M	H	I	I	F	M	I
	CH4	G	G	K	A	C	M	H	K	H	M	H	I	I	F	M	H
O. gorbuscha	PI1	H	G	L	A	C	N	I	L	I	N	H	K	K	G	N	I
	PI2	H	L	L	A	C	N	I	L	I	N	H	K	K	H	N	I

*Letters are restriction patterns (see Appendix A) for (from left to right) *Bam* HI, *Bgl* I, *Bgl* II, *Cfr* 42I, *Eco* RI, *Eco* 32I, *Eco* 81I, *Eco* 91I, *Eco* 105I, *Hinc* II, *Hind* III, *Nco* I, *Pvu* II, *Pst* I, *Sca* I and *Xba* I.

Appendix A). All taxa had a diagnostic genetic profile. However, in one sample from the zone of sympatry of *B. lenok* forms (the Ussuri basin) two out of 15 blunt-snouted lenoks had a genetic profile (BU3 clone) very similar (differ in loss of one *Hind* III restriction site only, $p=0{\cdot}17\%$) to the genetic profile of 15 sharp-snouted lenoks from the same locality (SU3 clone). The other 13 blunt-snouted lenoks had a BU1 composite genotype. It is likely that the BU3 clone was transferred from sharp- to blunt-snouted lenok by past introgressive hybridization events. No intraspecific variation was detected in *Hucho taimen* (Pallas) *Parahucho perryi* (Brevoort), *Oncorhynchus mykiss* (Walbaum), or *O. kisutch* (Walbaum).

The size of the mitochondrial genome of the analysed taxa was estimated to be approximately 16 700 bp, although intra-individual, as well as intra- and interpopulational variations of mtDNA size (increases) were detected in *Brachymystax* and *Hucho*. The intra- and interpopulation mtDNA size increases

TABLE III. Matrix of average % sequence divergence (p) among mtDNA genotypes observed for 11 salmonid taxa

	BS Lenok	SS Lenok	*H. tai*	*S. leu*	*S. mal*	*P. per*	*O. myk*	*O. kis*	*O. mas*	*O. ket*	*O. gor*
BS lenok	0·76										
SS lenok	2·24	0·34									
H. taimen	2·97	3·22	0·00								
S. leucomaenis	8·01	6·24	8·19	0·38							
S. malma	6·12	6·15	6·82	3·85	0·21						
P. perryi	7·08	6·73	6·54	7·65	7·12	0·00					
O. mykiss	9·04	8·24	9·41	7·29	8·79	6·86	0·00				
O. kisutch	8·19	9·06	8·35	9·19	8·22	6·44	3·27	0·00			
O. masou	7·25	7·69	8·95	7·60	6·88	5·98	5·15	4·70	0·20		
O. keta	9·33	9·36	9·41	9·55	8·91	7·10	5·78	4·70	6·80	0·28	
O. gorbuscha	7·95	7·65	9·00	8·09	8·21	7·63	6·11	5·39	6·00	3·38	0·30

BS and SS lenok, blunt-snouted and sharp-snouted forms of *B. lenok*, respectively.

ranged from 60 to 180 bp and from 60 to 300 bp, respectively (the details of the analysis of these data will be presented elsewhere).

Averaged mtDNA differences among taxa were highly variable (Table III). The *Oncorhynchus* species were highly divergent from the *Brachymystax* (p=7·25–9·36%), *Hucho* (p=8·35–9·41%) and *Salvelinus* (p=6·88–9·55%) species. The divergence between the *Brachymystax* and *Hucho* species was much smaller (p=2·97–3·22%) and did not exceed the level of congeneric variation within *Salvelinus* (p=3·85%) or *Oncorhynchus* (p=3·27–6·8%). The Sakhalin taimen (*Parahucho*) was almost equally divergent from the *Brachymystax* (p=6·74–7·08%), *Hucho* (p=6·35%), *Salvelinus* (p=7·12–7·65%) and *Oncorhynchus* (p=5·98–7·63%) species. The averaged mtDNA divergence estimate between blunt- and sharp-snouted lenoks was 2·24%, which is only slightly lower than the *Brachymystax–Hucho* divergence level. The mtDNA variation within taxa ranged from no detectable variation to 0·16–1·15% among populations of the two forms of lenok.

The UPGMA phenogram generated from the matrix of mtDNA divergences between 29 clones (Fig. 1) showed two main groupings: (1) *Brachymystax*, *Hucho* and *Salvelinus* species; and (2) *Parahucho* and *Oncorhynchus* species. The *Oncorhynchus* species were further divided into two subgroups: (1) *O. mykiss*, *O. kisutch* and *O. masou* (Brevoort), and (2) *O. keta* (Walbaum) and *O. gorbuscha* (Walbaum). The NJ tree (not presented) had a largely concordant topology. The major difference was that pairs of species (*O. mykiss* and *O. kisutch*) and (*O. keta* and *O. gorbuscha*) clustered first, and then *O. masou* joined to this cluster.

For the UPGMA clustering method, bootstrap analysis (Fig. 2) showed a high confidence (similar to conventionally accepted 95% significance level or above it) of the nodes only for closely related pairs of species: *Brachymystax lenok* (Pallas) and *H. taimen*; *Salvelinus malma* (Walbaum) and *S. leucomaenis* (Pallas); *O. mykiss* and *O. kisutch*; and *O. keta* and *O. gorbuscha*.

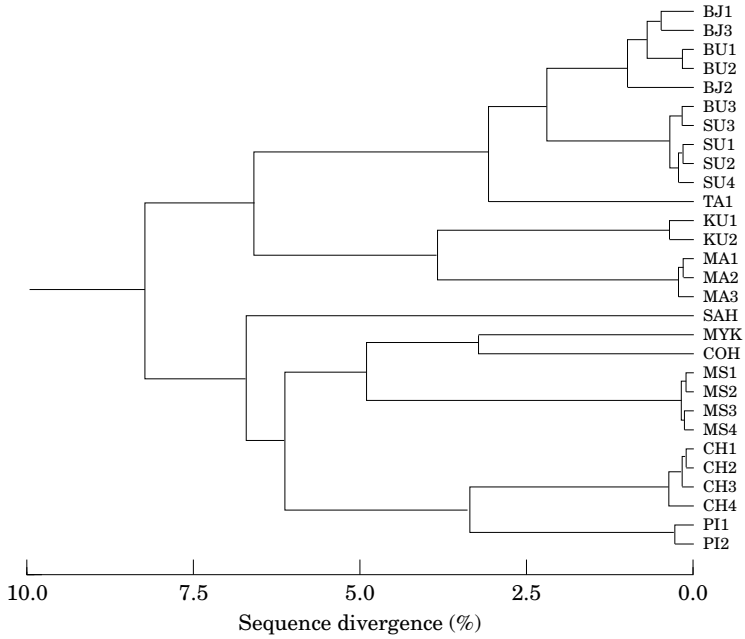

FIG. 1. UPGMA dendrogram summarizing sequence divergences among 29 composite mtDNA genotypes detected in 11 taxa of salmonid fishes (Table II, III).

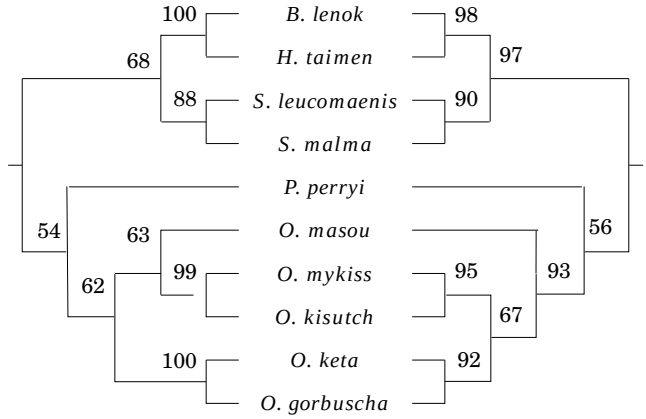

FIG. 2. 50% majority-rule consensus trees produced by bootstrap procedure when applying UPGMA (left) and NJ (right) clustering methods. Numbers at nodes are percentages of 100 bootstrapped replication in which the group projecting from each node appeared monophyletic. For simplicity of presentation, details of intraspecific branchings are omitted.

When applying the NJ tree-making algorithm, *Brachymystax*, *Hucho* and *Salvelinus* formed a distinct cluster in 97% of the bootstrap repeats. On the contrary, the grouping of *Parahucho* and *Oncorhynchus* was inconstant—this group occurred only 56 times out of 100 replicates. For the *Oncorhynchus* species an unresolved trichotomy was observed, which included the following

three groupings: (1) *O. mykiss*, *O. kisutch*; (2) *O. keta*, *O. gorbuscha*; and (3) *O. masou*.

The low percentage of the bootstrap repeats supporting the monophyly of *Parahucho* and *Oncorhynchus* grouping suggests that the root of the trees presented in Fig. 2 is a trichotomy of three evolutionary lines: (1) *Brachymystax*, *Hucho* and *Salvelinus*; (2) *Parahucho*; and (3) *Oncorhynchus*.

DISCUSSION

THE TAXONOMIC STATUS OF THE TWO FORMS OF LENOK

Currently, two complexes of forms (infraspecies) are distinguished within the genus *Brachymystax*—blunt- and sharp-snouted lenoks. These two forms differ significantly in the shape of their head and body, size of jaws, number of gill rakers, coloration, grounds of spawning, and other characters (reviewed in Mina, 1991). In all zones of sympatry (rivers of the Amur and Lena basins), the two forms of lenok are reproductively isolated, but hybridization sometimes occurs. The morphological characters differentiating the two forms display clinal variation, so that remote allopatric populations of the two forms prove to be similar (Alexeev *et al.*, 1986; Mina, 1991). For this reason these forms have not been described as separate species. However, recent studies of protein variation showed that: (1) blunt- and sharp-snouted lenoks are easily separated all over their geographical ranges (Nei's $D=0{\cdot}103$, range; 0·042–0·195); and (2) although hybridization occurs at some localities, the level of gene flow is very low (Osinov, 1993). Thus, according to the biological species concept, both forms should be given specific status (Osinov, 1993). Restriction analysis of mtDNA also detected traces of hybridization between the two forms of lenok, but the mtDNA divergence between them ($p=2{\cdot}24\%$) is only slightly below the divergence of *Hucho* and *Brachymystax* ($p=3{\cdot}10\%$). Consequently, it is possible to give the two forms of lenok species status.

THE TAXONOMIC RANK OF THE SAKHALIN TAIMEN

According to Holčik (1982), Holčik *et al.* (1988), and references therein, the genus *Hucho* consists of five species: the freshwater Danubian salmon *H. hucho* (L.), taimen *H. taimen*; Korean taimen *H. ishikawai* Mori; the poorly known Chinese taimen *H. bleekeri* Kimura; and the anadromous Sakhalin taimen *H. perryi*. The first two species are very similar and sometimes considered as subspecies. Apparently, *H. ishikawai* represents a derivative form of *H. taimen* (Holčik, 1982). As the Sakhalin taimen differs from the other *Hucho* species in many morphological and ecological characters, it has been assigned to a separate subgenus, *Parahucho* (Vladykov & Gruchy, 1972). The karyological studies (Anbinder *et al.*, 1982; Ráb & Liehman, 1982; Viktorovsky *et al.*, 1985; Cavender & Kimura, 1989; review—Hartley, 1987) have revealed essential differences between the Sakhalin taimen (the karyotype formula* is 38–42 msm, 20–24 ST+T, 2n=62, NF=100–104) on one hand, and *H. hucho* (30–32 msm, 50 ST+T, 2n=82, NF=112–114) and *H. taimen* (30–36 msm, 48–54 ST+T, 2n=84,

*msm, metacentric-submetacentric; ST, subtelocentric; T, telocentric chromosomes; 2n, diploid number; NF, arm number.

NF=114–120) on the other. The last two species are more similar to *B. lenok* (26–32 msm, 60–64 ST+T, 2n=90–92, NF=116–124), whereas the karyotype of the Sakhalin taimen is very similar to that found in some species of Pacific trouts especially the Yellowstone cutthroat trout *O. clarki bouvieri* Behnke (40 msm, 24 ST+T, 2n=64, NF=104). Based on these data, it has been suggested that the monotypic subgenus *Parahucho* should be raised to generic status (Anbinder *et al.*, 1982; Viktorovsky *et al.*, 1985). The recent electrophoretic data on protein variation in lenok, taimen and Sakhalin taimen have confirmed this opinion. The genetic divergence between taimen and lenoks (Nei's $D=0{\cdot}335\pm0{\cdot}107$) is much lower than that between taimen and Sakhalin taimen (Nei's $D=0{\cdot}755\pm0{\cdot}179$) (Osinov, 1991). According to the mtDNA data, *H. taimen* is also more similar to *B. lenok* ($p=3{\cdot}10\%$) than to the Sakhalin taimen ($p=6{\cdot}78\%$), thus supporting the separation of the Sakhalin taimen into a distinct genus.

MITOCHONDRIAL DNA *V.* MORPHOLOGICAL PHYLOGENY OF SALMONINAE

By tradition, the evolution of Salmoninae is viewed as successive offshoots of the genera *Brachymystax*, *Hucho*, *Salvelinus*, *Salmo* and *Oncorhynchus* from a common stem (Tchernavin, 1939; Norden, 1961). Accordingly, the freshwater *Brachymystax* was considered as the oldest and most primitive genus, whereas the anadromous *Oncorhynchus* was viewed as the youngest and most advanced genus.

The mtDNA distance data do not support this view of Salmoninae evolution. The intergeneric mtDNA divergence between freshwater *Brachymystax* and *Hucho* (Table III) is the lowest one found here, and these two genera must be considered as the youngest. In contrast, the genera including mainly anadromous species (*Oncorhynchus*, *Salmo*, *Parahucho* and *Salvelinus*) are much more divergent from each other (Table III; Berg & Ferris, 1984; Gyllensten & Wilson, 1987; Ginatulina *et al.*, 1988; Grewe *et al.*, 1990; McVeigh & Davidson, 1991; Shedlock *et al.*, 1992) and they should be considered as the ancient lineages. The fossil evidence suggests that Pacific salmons and trouts, taimens similar to *Parahucho*, and charrs could be as old as the late Miocene (reviews: Tomoda *et al.*, 1977; Smith, 1981; Cavallo & Gaudant, 1987). On the contrary, the application of the 1–1·5% sequence divergence per million year molecular clock for salmonid mtDNA (Shed'ko, 1991), would suggest that the ancestral forms of *Brachymystax* and *Hucho* diverged only about 2–3 million years ago, during the late Pliocene–early Pleistocene period. The similar divergence time between *Brachymystax* and *Hucho* was calculated using the allozyme molecular clock (approximately 1–2 million years ago; Osinov, 1991).

With respect to mtDNA data, both *Brachymystax* and *Hucho* are not the most divergent taxa among Salmoninae (Table III; Grewe *et al.*, 1990). According to morphological trees (e.g. Norden, 1961; Smith & Stearley, 1989) *Brachymystax* and *Hucho* are outgroups for all the other Salmoninae genera, whereas in the present trees they cluster with *Salvelinus* and this grouping occurred in 68–97% of the bootstrap repeats (Figs 1, 2). It should be noted, however, that these trees estimate the matriarchal genealogy of mtDNA, which is inherited as a block of genes without recombination. The topology of the given trees can differ from the

species tree due to: (1) the sampling errors when estimating the genetic divergence and the stochastic errors in the accumulation of substitution in DNA (Nei, 1987); (2) stochastic sorting of mtDNA lineages to daughter populations from a polymorphic ancestral population (Neigel & Avise, 1986; Pamilo & Nei, 1988); and (3) introgressive hybridization (e.g. Ferris *et al.*, 1983). In addition, owing to the specific character of raw data (see footnote in Material and Methods) and absence of a correct outgroup (*Coregonus* or *Thymallus* species), we could not use the more powerful phylogenetic methods (e.g. parsimony analysis; Hillis *et al.*, 1994). Therefore, it becomes difficult to say whether the observed phenogram (Fig. 1) represents a significant challenge to the view that the freshwater *Brachymystax* and *Hucho* are outgroups for all the anadromous genera of Salmoninae. To solve this contradiction, the phylogenetic trees of many independently transmitted genes, produced by the various tree-making methods (distance, parsimony, or maximum likelihood) must be compared.

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APPENDIX A

Fragment size estimates for all fragment patterns resulting from restriction analysis of salmonid mtDNA

Enzyme	Code letter	Fragment lengths (kilobase pairs)					Total
Bam HI	A	16·7*					16·7
	B	11·9	3·55	1·2			16·65
	C	10·3	5·0	1·4			16·7
	D	12·7	4·0				16·7
	E	10·5	5·0	1·2			16·7
	F	9·2	7·0	0·5			16·7
	G	9·2	6·4	0·6	0·5		16·7
	H	14·2	2·44				16·64
Bgl I	A	14·0	2·6				16·6
	B	7·62	6·8	2·6			17·02
	C	6·8	6·0	2·6	1·6		17·0
	D	11·2	3·0	2·6			16·8
	E	6·6	5·5	4·7			16·8
	F	11·6	5·2				16·8
	G	6·8	5·5	4·45			16·75
	H	5·5	4·5	4·45	2·3		16·75
	I	4·5	4·45	4·0	2·3	1·53	16·78
	K	11·0	4·45	1·32			16·77
	L	10·0	6·8				16·8
Bgl II	A	16·7*					16·7
	B	15·4	1·24				16·64
	C	10·0	7·4				17·4
	D	10·4	6·6				17·0
	E	6·6	6·3	4·1			17·0
	F	16·0	0·7				16·7
	G	12·1	4·7				16·8
	H	12·1	3·5	1·2			16·8
	I	9·6	3·5	2·5	1·2		16·8
	K	10·7	6·0				16·7
	L	10·7	3·5	2·5			16·7
Cfr 42I (*Sac* II)	A	12·8	2·1	1·8			16·7
Eco RI	A	8·1	7·8	0·76			16·66
	B	8·6	8·1				16·7
	C	7·8	4·05	4·05	0·76		16·66
	D	8·6	4·05	4·05			16·7
	E	12·7	4·05				16·75
Eco 32I (*Eco* RV)	A	8·0	3·8	3·4	1·08		16·28
	B	10·6	3·4	1·08			15·08
	C	8·65	3·8	3·4			15·85
	D	10·3	3·4	0·96	0·65		15·31
	E	11·2	1·8	1·2	0·8		15·0
	F	12·7	1·5	0·8			15·0

(*Continued*)

APPENDIX A
Continued

Enzyme	Code letter	Fragment lengths (kilobase pairs)								Total
	G	13·4	1·2	0·8						15·4
	H	16·0	0·8							16·8
	I	8·15	6·9	0·8						15·85
	K	14·1	1·0	0·8						15·9
	L	11·7	2·94	0·8						15·44
	M	7·95	3·23	2·8	1·15	0·8				15·93
	N	10·2	4·5	0·8						15·5
Eco 81I	A	8·05	4·7	2·0	1·35					16·1
(*Sau* I)	B	8·05	6·05	2·0						16·1
	C	12·8	2·0	1·35						16·15
	D	14·2	2·0							16·2
	E	7·4	3·5	2·8	2·0					15·7
	F	9·6	3·5	2·43	0·8					16·33
	G	11·2	3·5	2·0						16·7
	H	13·2	3·5							16·7
	I	11·2	2·15	2·0	1·4					16·75
Eco 91I	A	6·3	5·7	4·4	0·6					17·0
(*Bst* EII)	B	10·1	6·3	0·6						17·0
	C	9·1	6·3	0·7	0·7	0·6				17·4
	D	6·1	5·86	3·3	1·2	0·6				17·06
	E	11·8	3·3	1·2	0·6					16·9
	F	9·1	4·6	2·3	0·6					16·6
	G	13·8	2·6	0·6						17·0
	H	16·02	0·6							16·8
	I	6·0	4·6	2·6	1·7	1·3	0·6			16·8
	K	7·1	4·6	3·1	1·7					16·5
	L	4·6	4·0	3·3	2·9	1·3	1·0			17·1
Eco 105I	A	11·9	5·0							16·9
(*Sna* BI)	B	8·5	5·0	1·43	1·1	0·5				16·53
	C	8·5	5·0	2·57	0·5					16·57
	D	6·8	5·83	3·7	0·5					16·83
	E	5·83	3·7	2·9	2·0	1·43	0·5			16·36
	F	8·2	5·83	2·0	0·5					16·53
	G	5·65	4·31	4·13	2·0	0·5				16·59
	H	5·83	4·46	2·57	2·0	1·15	0·5			16·51
	I	8·5	4·46	2·0	1·15	0·5				16·61
Hinc II	A	4·93	3·55	3·33	3·33	0·76	0·6			16·5
	B	4·93	3·55	3·33	1·74	1·74	0·76	0·6		16·65
	C	5·51	3·33	3·33	3·23	0·76	0·6			16·76
	D	4·1	3·0	2·75	2·55	0·93	0·85	0·76	0·6	15·54
	E	4·1	3·1	2·55	1·75	1·3	0·93	0·76	0·6	15·09
	F	4·1	3·1	3·0	2·55	0·93	0·76	0·6		15·04
	G	3·86	3·86	3·44	3·25	0·93	0·85	0·76		16·95
	H	5·6	4·1	3·6	2·75	0·93				16·98
	I	4·8	2·75	2·45	2·42	1·1	0·93			14·45

(*Continued*)

APPENDIX A
Continued

Enzyme	Code letter	Fragment lengths (kilobase pairs)								Total
	K	4·2	3·6	3·4	2·55	1·8	0·85			16·4
	L	4·2	3·4	2·75	2·55	1·8	0·93	0·85		16·48
	M	4·0	3·28	3·28	2·3	1·52	0·76	0·63		15·77
	N	3·8	3·28	2·85	2·85	1·7	1·28	0·63		16·39
Hind III	A	8·8	3·5	2·3	1·8	0·3				16·7
	B	8·8	3·5	1·8	1·21	1·1	0·3			16·71
	C	5·0	3·8	3·5	1·8	1·21	1·1	0·3		16·71
	D	5·0	3·8	3·5	2·3	1·39	0·41	0·3		16·7
	E	6·6	3·5	2·2	1·8	1·21	1·1	0·3		16·71
	F	6·6	3·5	2·3	2·2	1·8	0·3			16·7
	G	3·8	3·5	2·8	2·3	2·2	1·8	0·3		16·7
	H	5·0	3·5	2·3	2·2	1·8	1·4	0·3	0·2	16·7
Nco I	A	8·3	4·4	2·7	1·5					16·9
	B	8·3	4·4	4·2						16·9
	C	8·3	4·4	2·7	0·9	0·6				16·9
	D	7·0	6·4	1·7	1·05	0·8				16·95
	E	8·3	4·4	3·65	0·73					17·08
	F	7·75	5·73	2·7	0·66					16·84
	G	5·73	3·65	2·75	2·7	1·24	0·66			16·73
	H	8·3	4·0	3·65	0·66					16·61
	I	14·8	1·24	0·66						16·7
	K	8·3	7·75	0·66						16·71
Pvu II	A	9·33	3·4	2·4	1·4					16·53
	B	6·8	3·4	2·6	2·4	1·4				16·6
	C	6·8	6·0	3·9						16·7
	D	9·33	6·0	1·4						16·73
	E	8·6	3·4	2·6	1·4	0·8				16·8
	F	10·6	3·4	2·6						16·6
	G	6·8	4·8	2·6	2·4					16·6
	H	6·8	3·4	2·6	2·4	1·4				16·6
	I	6·8	4·8	2·4	2·3	0·3				16·6
	K	6·8	3·4	2·4	2·3	1·4	0·3			16·6
Pst I	A	16·7*								16·7
	B	12·9	3·8							16·7
	C	12·6	4·1							16·7
	D	10·5	4·1	1·7	0·4					16·7
	E	15·6	1·2							16·8
	F	14·7	2·1							16·8
	G	11·8	2·9	2·1						16·8
	H	10·5	4·1	2·1						16·7
Sca I	A	6·4	5·7	5·2						17·3
	B	6·8	4·7	4·2	1·3					17·0
	C	11·4	5·7							17·1
	D	5·2	4·85	4·7	1·9	0·3				16·95
	E	6·0	5·7	5·2	0·4					17·3

(*Continued*)

APPENDIX A
Continued

Enzyme	Code letter	Fragment lengths (kilobase pairs)						Total
	F	6·6	5·3	4·7	0·3			16·9
	G	11·7	5·0					16·7
	H	5·2	5·0	5·0	0·8	0·6		16·6
	I	4·7	4·5	3·5	1·3	1·1	1·0	16·1
	K	8·7	6·9					15·6
	L	10·6	6·4					17·0
	M	10·6	3·0	1·65	1·3			16·55
	N	5·6	4·8	4·7	1·3	0·5		16·9
Xba I	A	9·9	3·5	3·3	0·2			16·9
	B	9·9	3·7	3·3				16·9
	C	13·5	3·3					16·8
	D	7·6	6·0	3·3				16·9
	E	5·1	4·7	3·7	3·3			16·8
	F	5·1	3·7	3·3	2·5	1·55	0·73	16·88
	G	7·6	3·7	3·3	1·55	0·73		16·88
	H	5·1	3·7	3·3	2·5	2·3		16·9
	I	7·6	3·7	3·3	2·3			16·9

*Single cut site.