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EXPERIMENTAL ARTICLES

Phylogenetic Relationships of Sakhalin Taimen *Parahucho perryi* Inferred from PCR–RFLP Analysis of Mitochondrial DNA

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Abstract—RFLP analysis of three amplified mtDNA fragments (Cyt*b*/D-loop, ND1/ND2, and ND3/ND4L/ND4) was performed in the following taxa: *Parahucho perryi*, *Hucho taimen*, *Brachymystax lenok*, *B. tumensis*, *Salmo salar*, *Salvelinus leucomaenis*, and *S. levanidovi*. For mtDNA of *P. perryi*, a substantial decrease in the haplotype and nucleotide diversity was observed as a result of random genetic drift, caused by a reduction in the effective population size. Nucleotide divergence estimates between the mtDNA haplotypes were determined. Sakhalin taimen *P. perryi* was found to be approximately equally diverged from *S. salar* and from the charrs of the genus *Salvelinus*, by 11.0 and 10.0%, respectively. The divergence between *P. perryi* and *H. taimen* constituted 14.6%, between *P. perryi* and lenoks of the genus *Brachymystax*, 14.2%, and between *H. taimen* and *Brachymystax*, 7.7%. The analysis of possible phylogenetic relationships of the mtDNA from *P. perryi* among the group of taxa examined confirmed validity of the genus *Parahucho*. Phylogenetic reconstructions performed showed that robustness of the trees constructed for the complex of phylogenetically informative characters over three mtDNA fragments was considerably higher than that of the trees constructed for individual genes.

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INTRODUCTION

Investigations of small-sized species with limited distribution ranges usually meet certain difficulties. Sakhalin taimen *Parahucho peryi* is one of such species. This species is the least investigated and the most disputable taxon among the family Salmonidae. Based on morphological, ecological and geographic distribution data, Sakhalin taimen was isolated in a separate subgenus *Parahucho* [1–8]. Karyotype analysis of lenoks and taimens confirmed the unique position of *P. perryi*. As a result, it was suggested to treat *Parahucho* as a genus [9]. Simultaneously, a hypothesis was advanced, according to which *P. perryi* was the most ancient form of modern salmonids, the unique representative of the subfamily Parahuchoninae [6]. This conclusion was not generally accepted, and phylogenetic position of *P. perryi*, as well as possible paraphyly of the genus *Hucho* are still a matter of considerable debate [5, 7, 10, 11]. The latter problem appeared when the data demonstrating that members of genera *Hucho* and *Brachymystax* were closer to each other than those of *Hucho* and *Parahucho* became available [1, 4, 6, 11]. In this case, inclusion of *P. perryi* (even at the subspecies rank) in the genus *Hucho* results in the formation of paraphyletic taxon.

Analysis of mtDNA sequences enlarged the possibilities for phylogenetic analysis, including the taxa of different ranks of the family Salmonidae. At the same time, mtDNA of *P. perryi* still remains poorly investigated [12–15]. The ideas on intraspecific genetic variation of *P. perryi* are limited to two studies on allozyme analysis [4, 16]. In this respect, possible future application of the analysis of microsatellite loci polymorphisms seems to be promising [17].

The present study was focused on the analysis of intraspecific variation of mtDNA along with possible phylogenetic relationships of Sakhalin taimen *P. perryi* based on PCR–RFLP analysis of three mtDNA fragments (Cyt*b*/D-loop, ND1/ND2, and ND3/ND4L/ND4). Considering the fact that most of the mitochondrial genes located in these fragments were never investigated in *P. perryi*, the data obtained are of interest, primarily for understanding evolutionary possibilities of adaptation of the species.

MATERIALS AND METHODS

We used material from the authors, collection gathered in different years in the rivers of the Russian Far East. The mtDNA samples from the following taxa were examined: Sakhalin taimen *Parahucho perryi* (Brevoort), taimen *Hucho taimen* (Pallas), sharp-snouted lenok *Brachymystax lenok* (Pallas) (according to [18]), blunt-snouted lenok *Brachymystax tumensis* (Mori) (according to [18]), Atlantic salmon *Salmo salar* (L.), white spotted charr *Salvelinus leucomaensis* (Pallas), and Levanidov charr *Salvelinus levanidovi* Chereshnev, Scopetz, Gudkov (Table 1).

Table 1. Main characteristics of the samples examined

Species	Population	Latitude	Longitude	Sample size
Parahucho perryi	Kievka River (western coast of the Sea of Japan)	43°06′N	134°17′E	6
P. perryi	Tumnin River (western coast of the Sea of Japan)	49°11′N	140°21′E	10
P. perryi	Maksimovka River (western coast of the Sea of Japan)	46°05′N	137°54′E	3
Hucho taimen	Manoma river (Amur River basin)	49°21′N	137°24′E	2
H. taimen	Arichi River (Amur River basin)	49°27′N	136°50′E	5
Brachymystax lenok	Manoma river (Amur River basin)	49°21′N	137°24′E	2
B. lenok	Arichi River (Amur River basin)	49°27′N	136°50′E	2
Brachymystax tumensis	Manoma river (Amur River basin)	49°21′N	137°24′E	5
B. tumensis	Anyui River (Amur River basin)	49°17′N	137°55′E	1
B. tumensis	Arichi River (Amur River basin)	49°27′N	136°50′E	1
Salmo salar	Tessema River (Taimyr Penninsula)	77°19′N	102°31′E	3
Salvelinus leucomaenis	Izmena Bay, Kunashir Island	43°43′N	145°28′E	10
Salvelinus levanidovi	Yama River (northern coast of the Sea of Okhotsk)	59°41′N	154°06′E	13

Individual preparations of total DNA were extracted from fixed liver and heart tissues according to a standard technique [19]. The mtDNA variation was examined using restriction analysis of three fragments amplified in polymerase chain reaction. One of these fragments included the cytochrome *b* gene and a regulatory region (Cyt*b*/D-loop), and the other two fragments encoded five subunits of NADH dehydrogenase (ND1/ND2, ND3/ND4L/ND4). The primers for the mtDNA fragments examined, their positions on the mtDNA map, and amplification conditions are described in [20]. Each of the amplified fragments was examined using a set of 17 restriction endonucleases in the conditions recommended by the manufacturers (MBI Fermentas, Lithuania and SibEnzim, Russia): *Asu*I, *Ava*I, *Ava*II, *Bst*NI, *Bst*UI, *Bsu*RI, *Dde*I, *Hin*fI, *Hha*I, *Mbo*I, *Mbo*II, *Msp*I, *Rsa*I, *Ssp*I, *Sty*I, *Taq*I, and *Vsp*I. A detailed method of the analysis was described earlier [21].

For phylogenetic analysis, four data matrices were formed, including individual matrices for each of the three amplified fragments and a pooled matrix for mtDNA as a whole. Binary matrices of the restriction site presence/absence for the combined haplotypes were constructed using the GENERATE software program; repeated haplotypes were grouped using the GROUP computer program within the REAP package [22]. Genetic distances were calculated using standard and modified estimates of nucleotide divergence between the mtDNA haplotypes [23, 24]. The estimates obtained were used for phylogenetic analysis with the help of the unweighted pair-group method with arithmetic average (UPGMA [25] and neighbor-joining method (NJ [26]). Cluster robustness was tested using bootstrap analysis with 1000 random permutations [27]. Nucleotide divergence, haplotype clusterization, and dendrogram topologies were evaluated using the PHYLIP 3.67 phylogenetic package [28] (the RESTDIST, SEQBOOT, NEIGHBOR, and CONSENSE programs) (http://evolution.qs.washington.edu/phylip.html).

Phylogenetic analysis was performed using the method of maximum parsimony (MP) for each set of charactes with the help of the PAUP version 4.0b10 [29] and PHYLIP 3.67 software packages. Within the PAUP package, the program performed a heuristic search of most parsimonious tree (the TBR and MulTrees options) in 100 reiterations with the limitation of maximum possible tree number to 1000. Robustness of the branching nodes obtained was tested using nonparametric bootstrap analysis with 1000 random permutations for each data set. In cases, when the best tree was impossible to find, the differences between alternative topologies were evaluated using the Templeton test [30] (the PAUP software package). A search for most parsimonious tree was performed using the branch and bound algorithm in the PENNY software program [31] among the 1000 pseudoreplicates obtained from the initial matrix within the SEQBOOT program (PHYLIP 3.67). Graphic images of all dendrograms were obtained using the TreeView program (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

The main genetic variation indices were determined for each of the amplified fragments and for the entire mtDNA using the REAP and ARLEQUIN vers. 2.000 software packages [32]. The interpopulation polymorphism was evaluated through the calculation of means of the differences between the haplotype pairs in the sample [33] and of nucleotide (π) and haplotype (h)

Table 2. Comparative characteristics of the mtDNA variation indices: analysis of the Cyt*b*/D-loop, ND1/ND2, and ND3/ND4L/ND4 regions

Species	Number of individuals	Number of haplotypes	Number of polymorphic sites	Between-haplotype differences estimate	Haplotype diversity *h*	Nucleotide diversity π
P. perryi	19	3	2	0.6199 ± 0.5103	0.5731 ± 0.0614	0.00108
H. taimen	7	4	7	3.0476 ± 1.8005	0.8095 ± 0.1298	0.00531
B. lenok	4	4	12	6.3333 ± 3.8011	1.0000 ± 0.1768	0.01103
B. tumensis	7	7	31	10.000 ± 5.2131	1.0000 ± 0.0764	0.01742
S. salar	3	2	3	2.0000 ± 1.5185	0.6667 ± 0.3143	0.00348
S. leucomaenis	10	4	14	3.4222 ± 1.9091	0.5333 ± 0.1801	0.00596
S. levanidovi	13	8	24	7.7948 ± 3.8822	0.8846 ± 0.0699	0.01358

Table 3. Comparative characteristics of variation in three mtDNA regions of *P. perryi*

MtDNA region	Number of individuals	Number of haplotypes	Number of sites	Polymorphic sites	Between-haplotype differences estimate	Haplotype diversity *h*	Nucleotide diversity π
Cyt*b*/D-loop	19	3	208	2	0.6199 ± 0.5103	0.5731 ± 0.0614	0.00298
ND1/ND2	19	1	199	0	0.0000 ± 0.0000	0.0000 ± 0.0000	0.00000
ND3/ND4L/ND4	19	1	167	0	0.0000 ± 0.0000	0.0000 ± 0.0000	0.00000

diversity [34, 35]. Heterogeneity of haplotype frequencies in the *P. perryi* samples was evaluated with the help of the Monte Carlo method with 10000 pseudoreplicates [36].

RESULTS AND DISCUSSION

Comparative Analysis of Intraspecific Variation in Sakhalin Taimen P. perryi

The sizes of the amplified mtDNA fragments were equal to 7814 bp. The total number of sites identified based on the analysis of the fragment sizes and the distribution of restriction fragments among the haplotypes was 574, corresponding to 2300 nucleotide bases, or 13.8% of the mitochondrial genome. In 63 individual fish examined, a total of 32 mtDNA haplotypes were identified. No common haplotypes for the taxa examined were found. Comparative characteristics of the variation indices are presented in Table 2.

Among the taxa investigated, *P. perryi* was characterized by the lowest genetic diversity indices. Haplotype composition of the Sakhalin taimen sample was represented by three haplotypes with the average estimate of the pairwise differences between them lower than 1. It is evident that the low number of nucleotide substitutions between the haplotypes determined the low indices of genetic diversity (0.00108) in the pooled sample of Sakhalin taimen (Table 2). Analysis of the variation distribution within the *P. perryi* genome part examined showed that polymorphism was determined by the Cyt*b*/D-loop fragment. The ND1/ND2 and ND3/ND4L/ND4 fragments were found to be completely monomorphic (Table 3).

Low genetic diversity indices in case of *S. leucomaenis* (Table 3) were related to sampling error, and the estimates obtained probably did not reflect the real mtDNA diversity [37]. At the same time, low diversity estimates obtained for *P. perryi* cannot be completely the small sample size. The diversity were comparable with those obtained earlier based ob RFLP analysis of mtDNA [12]. In addition, the decrease of genetic diversity in Sakhalin taimen was observed not only for the mitochondrial, but also for the nuclear genes [4, 16]. Allozyme analysis showed that *P. perryi* was characterized by extremely low values of mean heterozygosity, proportion of polymorphic loci, and the mean number of alleles per locus. Only in one out of four *P. perryi* populations examined, polymorphism at a single locus was detected.

The loss of genetic variation was thoroughly studied at the example of those fish species, which were subjected to acclimatization and artificial reproduction [38]. In natural populations, there are few examples of such phenomenon. Almost complete absence of mtDNA variation was observed in the *Salvelinus* charrs as a consequence of the random genetic drift in the conditions of geographic isolation [39–41]. We think that the decrease of genetic diversity in the *P. perryi* populations is also caused by random genetic drift. It is known that intensity of the genetic drift is inversely related to the genetically effective population size (N_e). Furthermore, N_e is usually smaller than absolute population size and reproduction number [38]. It should be noted that absolute monomorphy of nuclear genomes was described for such large mammals, such as polar bear [42] and elephant seal [43]. It is suggested that low heterozygosity in these species is associated with effec-

tive population size, which can vary during long species evolution under the influence of the following factors: local extinction of individual populations, population number variation between the generations, sex ratio, etc. [44].

At present, Sakhalin taimen has a limited range, which does not extend far from the ancestral water basin (the present-day Sea of Japan). The species is characterized by high morphoecological homogeneity [45]. Some specific biological features of *P. perryi* (high growth rate, late maturation, and low reproduction capacity) maintain relatively low population number. Furthermore, absolute homing typical of the species [46] prevents migration, which could to some extend compensate the effects of the gene drift. Combination of these characters in historical context determined the low polymorphism of the *P. perryi* genome. Analysis of the samples heterogeneity relative to the haplotype frequencies performed with the help of the Monte Carlo approach [36] revealed statistically significant differences between the *P. perryi* population pairs, namely, the populations from Kievka and Tumnin rivers ($\chi^2 = 8.65$; $d.f. = 2.14$; $P = 0.001$), as well as from Tumnin and Maksimovka rivers ($\chi^2 = 4.24$; $d.f. = 2.18$; $P = 0.01$). Taking into consideration the minimum number of nucleotide substitutions between the *P. perryi* mtDNA haplotypes (Table 2), these haplotypes can be considered those of a single ancestral population. It is suggested that the change of genetic characteristics under the influence of random genetic drift occurs independently in each population, explaining the heterogeneity revealed.

Genetic Divergence

Pairwise estimates of nucleotide divergence between the mtDNA haplotypes [23], calculated for the Cyt*b*/D-loop, ND1/ND2, and ND3/ND4L/ND4 complex, were determined. It was demonstrated that Sakhalin taimen was almost equally diverged from *S. salar*, as well as from the charrs of the genus *Salvelinus*, by 11.0 and 10.0%, respectively. Higher divergence was observed upon comparison of the *P. perryi* haplotypes with taimen and lenoks. Divergence between *P. perryi* and *H. taimen* was 14.6%, and between *P. perryi* and lenoks of the genus *Brachymystax* it was 14.2%, while between *H. taimen* and *Brachymystax* it was 7.7%. The estimates obtained were comparable with the divergence between the *cytb* haplotypes of the genera *Parahucho*, *Salvelinus*, and *Salmo* (11.0 to 11.2%) [8]. At the same time, these estimates were considerably higher than those obtained based on the RFLP analysis of mtDNA [13].

Comparison of the genetic distances between the *Parahucho* and *Hucho* taimens and the *Brachymystax* lenoks presents certain difficulties, since these taxa are unsufficiently studied genetically. Only the divergence estimates based on allozyme analysis [4], RFLP analysis of mt DNA [13], ribosomal DNA (rDNA) data [10], the growth hormone 2 gene intron C (GH-IIC) [11], and the mtDNA *cytb* [8] are available in literature. Nevertheless, a common trend can be traced. It was demonstrated that irrespectively of the genetic divergence measures used, the divergence between *Parahucho* and *Hucho*, as well as between *Parahucho* and *Brachymystax* were approximately 2 to 2.5 times higher than between *Hucho* and *Brachymystax*. This tendency was also observed in PCR–RFLP analysis of the Cyt*b*/D-loop, ND1/ND2, and ND3/ND4L/ND4 segments of mitochondrial genome.

The *P. perryi – S. leucomaenis* pair deserves special interest. Calculation of the pairwise distances between the mtDNA haplotypes using the principle of mutation steps minimization [47], showed that the *P. perryi* haplotypes were most close to the haplotypes of *S. leucomaenis* (minimal distance constituted 216 mutation steps). However, taking into consideration the homoplasy level (30 for this pair, on average) it is too early to draw a conclusion on the genetic similarity *P. perryi* and *S. leucomaenis*. At the same time, according to the data on the RFLP analysis of rDNA in the group of taxa of different ranks, the lowest differences were observed between *P. perryi* and *S. leucomaenis* (2.23%). This was 1.5 to 2 times lower than the divergence between *P. perryi* and the two charr species (*S. fontinalis* and *S. namaycush*) [10].

Phylogenetic Analysis

Phylogenetic analysis was performed separately for each amplified fragment, as well as for the mtDNA data set as a whole. Dendrograms (NJ and MP) were constructed using genetic distance matrices phylogenetically informative characters. Since at the present step of investigation no task to determine the succession of the taxa divergence was set, the outer group was not included into the analysis, and all the trees presented are unrooted. Depending on the character set, the phylogenetic schemes constructed differed by the sequence of clustering, content of the forming clusters, and the branching nodes robustness (Figs. 1–4).

Cytb/D-loop fragment. The Cyt*b*/D-loop matrix included 208 sites, of which 180 were determined as phylogenetically informative, or shared derived characters. According to the topology of the NJ tree (Fig. 1a) constructed for the character complex examined, haplotypes of *Salvelinus* and *Parahucho* formed a well-supported cluster (99%), and the *Salmo* haplotype was attached to this cluster (63%). Bootstrap analysis also supported grouping of *Hucho* and *Brachymystax* haplotypes in one cluster (100%). However, due to low estimates of subsequent branching, the position of *Hucho*, *B. lenok*, and *B. tumensis* should be considered as trichotomy. The position of *Salmo* haplotype is considered as extremely weakly supported (63%). Phylogenetic analysis performed using maximum parsimony approach with two calculation models did not yield a single most parsimonious tree. Using branch and bound

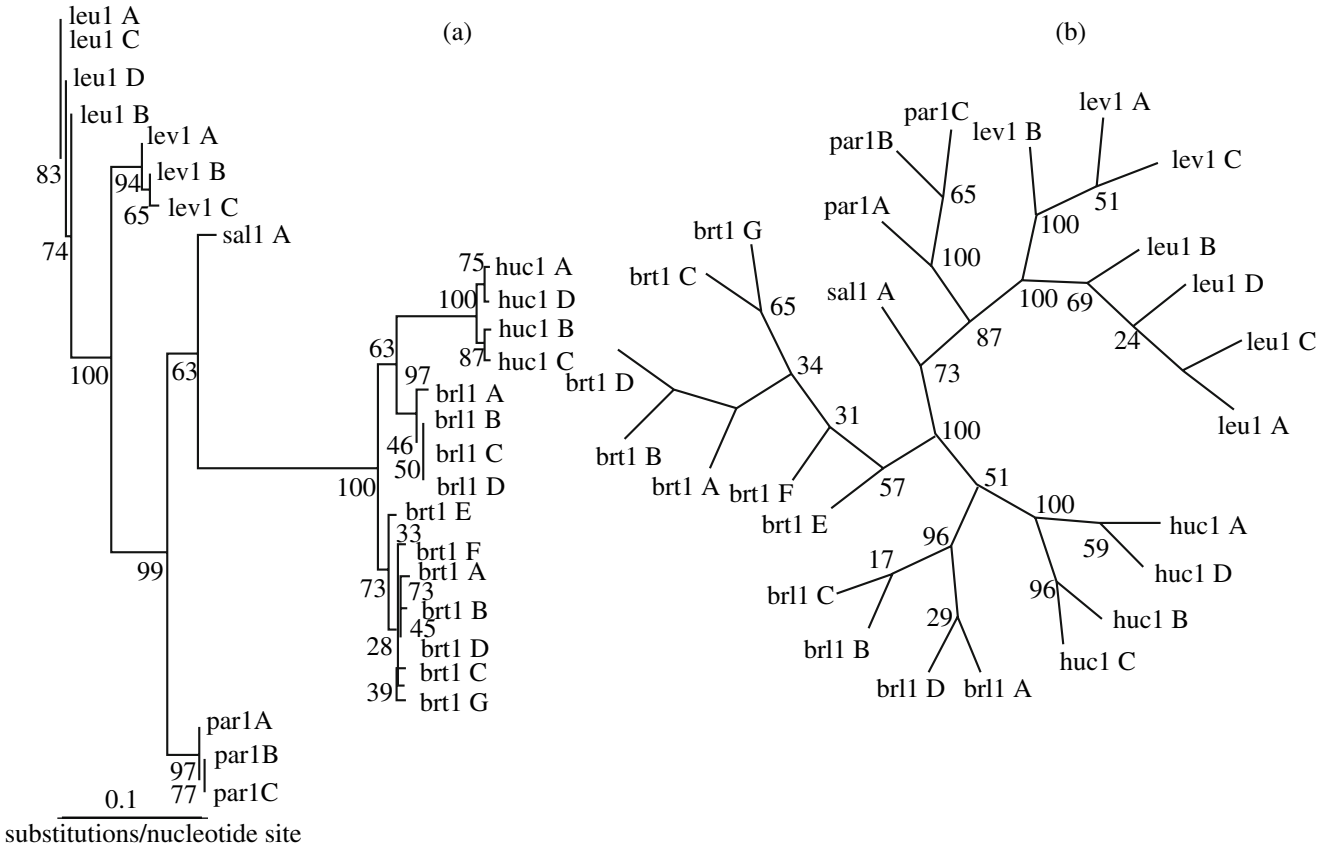

Fig. 1. Comparison of the mtDNA phylogenetic analysis data (the Cyt*b*/D-loop fragment, 180 phylogenetically informative characters): a, NJ-tree; b, MP-tree. Haplotype designations: *P. perryi*, par1; *H. taimen*, huc1; *B. lenok*, brl1; *B. tumensis*, brt1; *S. salar*, sal1; *S. leucomaensis*, leu1; *S. levanidovi*, lev1. The figures are bootstrap values (in % from 1000 replicates).

option of the PENNY program, a total of 75 trees were constructed (tree length, 292 mutation steps), and a heuristic search performed within the PAUP program resulted in 40 trees. The trees constructed differed only by the branching orders within the clusters of *Brachymystax* haplotypes. Since the maximum parsimonious tree is shorter (tree length, 273 steps; consistency indices, CI, 0.659; RC, 0.603; retention index, RI, 0.915; homoplasy index, HI, 0.341), its topology (Fig. 1b) is presented in accordance to the PAUP program. The differences among 40 trees, evaluated with the help of Templeton test [30], were not statistically significant, because relative to the main characteristics, these trees did not differ from the maximum parsimonious tree ($P = 1.00$). However, the maximum parsimonious tree topology was weakly supported (Fig. 1b). Bootstrap analysis supported monophyly of the *Salvelinus* (100%), *Hucho* and *Brachymystax* (100%) haplotypes. The *Parahucho* branch occupied an outer position relative to the monophyletic *Salvelinus* group. The *Salmo* branch was placed closer to the monopyletic *Hucho–Brachymystax* group. Compared to the NJ tree, the bootstrap support value of the *Parahucho* branch was substantially decreased (87%), while it was slightly higher for the *Salmo* branch (73%). Thus, position of both branches remained unresolved due to weak support of the branching nodes.

ND1/ND2 fragment. The ND1/ND2 matrix included 132 phylogenetically informative characters from 199 sites. According to the NJ tree topology (Fig. 2a) constructed for this character set, the haplotypes formed three clusters, namely, *S. leucomaenis* and *S. levanidovi, Hucho* and *Brachymystax,* and *Parahucho* and *Salmo*. Due to low bootstrap support, the position of three branches (*Parahucho*, *Salmo*, and the *Hucho–Brachymystax* common branch) should be considered as trichotomy. Phylogenetic analysis performed using the method of maximum parsimony with two calculation models yielded the same maximum parsimonious tree (tree length, 205 steps; CI, 0.644; RC, 0.516; RI, 0.804; and HI, 0.356). Statistical estimate of nonrandom tree topology pattern was low (Fig. 2b). Strong bootstrap support (100%) was obtained for the branching nodes of the *Salvelinus*, *Brachymystax* and *Hucho–Brachymystax* haplotypes. The *Parahucho* branch occupied an outer position relative to the monophyletic *Salvelinus* group, while the *Salmo* branch was located closer to the well-supported *Hucho–Brachymystax* group. At the same time, position of *Parahucho*

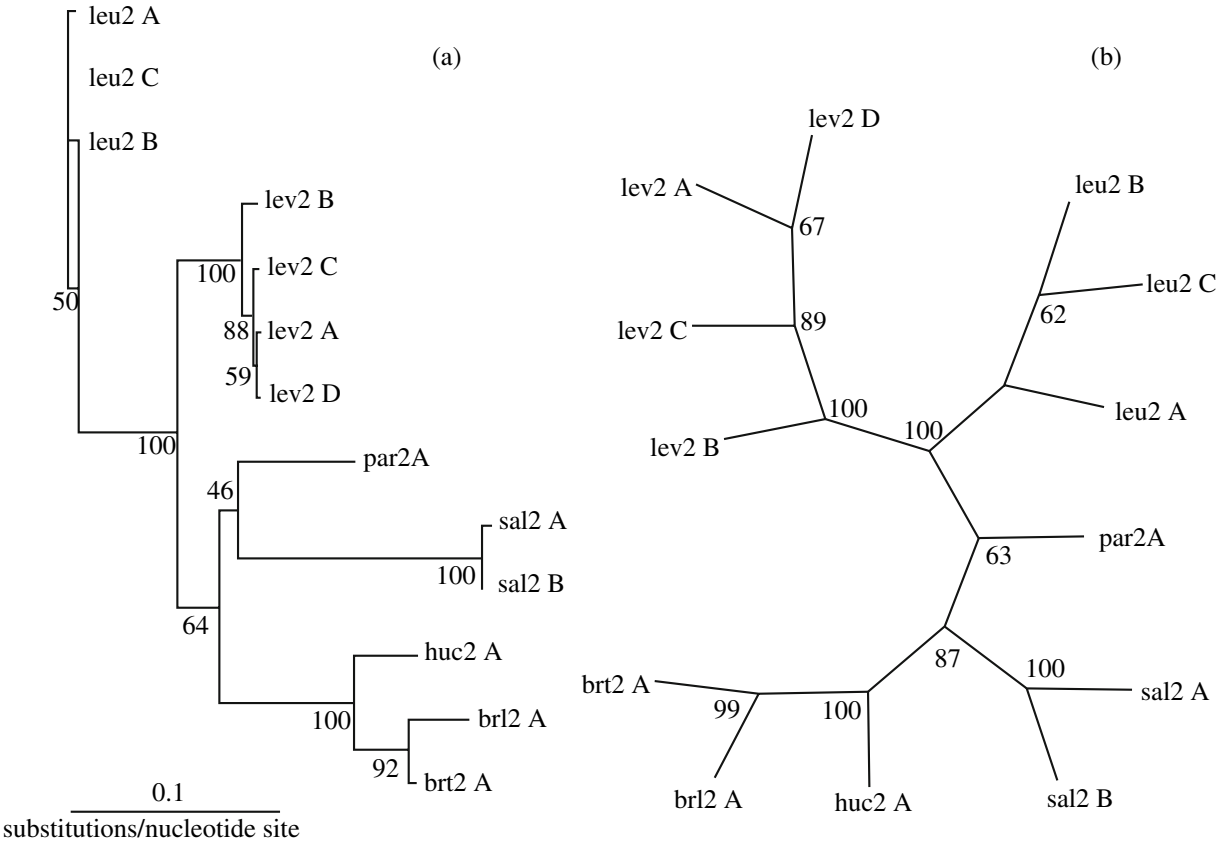

Fig. 2. Comparison of the mtDNA phylogenetic analysis data (the ND1/ND2 fragment, 132 phylogenetically informative characters): a, NJ-tree; b, MP-tree. Haplotype designations: *P. perryi*, par2; *H. taimen*, huc2; *B. lenok*, brl2; *B. tumensis*, brt2; *S. salar*, sal2; *S. leucomaensis*, leu2; *S. levanidovi*, lev2. The figures are bootstrap values (in % from 1000 replicates).

remained unresolved due to low branching node bootstrap value (63%).

ND3/ND4L/ND4 fragment. The ND3/ND4L/ND4 matrix included 124 phylogenetically informative characters from 167 sites. According to the NJ tree topology (Fig. 3a) constructed for this character set, haplotypes of *Salvelinus* and *Salmo* formed a well-supported cluster (96%). Haplotype of *Hucho* clustered together with *Brachymystax* (70%), while *Parahucho* occupied an intermediate position between the *Hucho* and *Salmo* branches (75%). Phylogenetic analysis performed using maximum parsimony approach with two calculation models did not yield a single most parsimonious tree. A total of nine trees (tree length, 241 steps) were constructed using the branch and bound option of the PENNY program, and heuristic search performed with the PAUP package yielded four trees. The trees differed only by the branching order within the *S. levanidovi* and *Brachymystax* clusters. The differences among all trees [30] were not statistically significant ($P = 1.00$). Topology of more parsimonious MP tree (Fig. 3b) presented in accordance to the PAUP package (tree length, 205 steps; CI, 0.605; RC, 0.505; RI, 0.834; and HI, 0.395) is similar to that of the NJ tree. According to the phylogenetic hypothesis suggested *Salmo* occupies a well-supported position of the outer branch relative to the *Salvelinus* monophyletic group (100%). Monophyly of the *Brachymystax* was also supported (100%). The *Parahucho* occupied an outer position relative to the *Hucho-Brachymystax* group; however bootstrap values for the *Parahucho* and *Hucho* branching nodes were lower than for the NJ-tree ((Fig. 3).

Cytb/D-loop, ND1/ND2, ND3/ND4L/ND4 fragment. The total set of characters consisted of 574 sites, 536 of which were phylogenetically informative. Since the hypothesis on the constant rate of the taxon evolution cannot be completely rejected on the basis of on the genetic distance estimates between the mtDNA haplotypes, for the set containing 574 sites, an UPGMA phenogram was constructed (Fig. 4a). An NJ tree was constructed for the set of 536 phylogenetically informative characters (Fig. 4b). The topology of the trees was similar, differing only by the branch lengths for the combined clusters, and the branch robustness for the clusters of *Parahucho* and *Salmo* haplotypes. It should be noted that haplotypes statistically significantly (100% significance level) grouped in accordance with their taxonomic affiliation. The *Hucho–Brachymystax* and *S. leucomaenis–S. levanidovi* clusters were well-supported. According to the UPGMA-tree topology, clus-

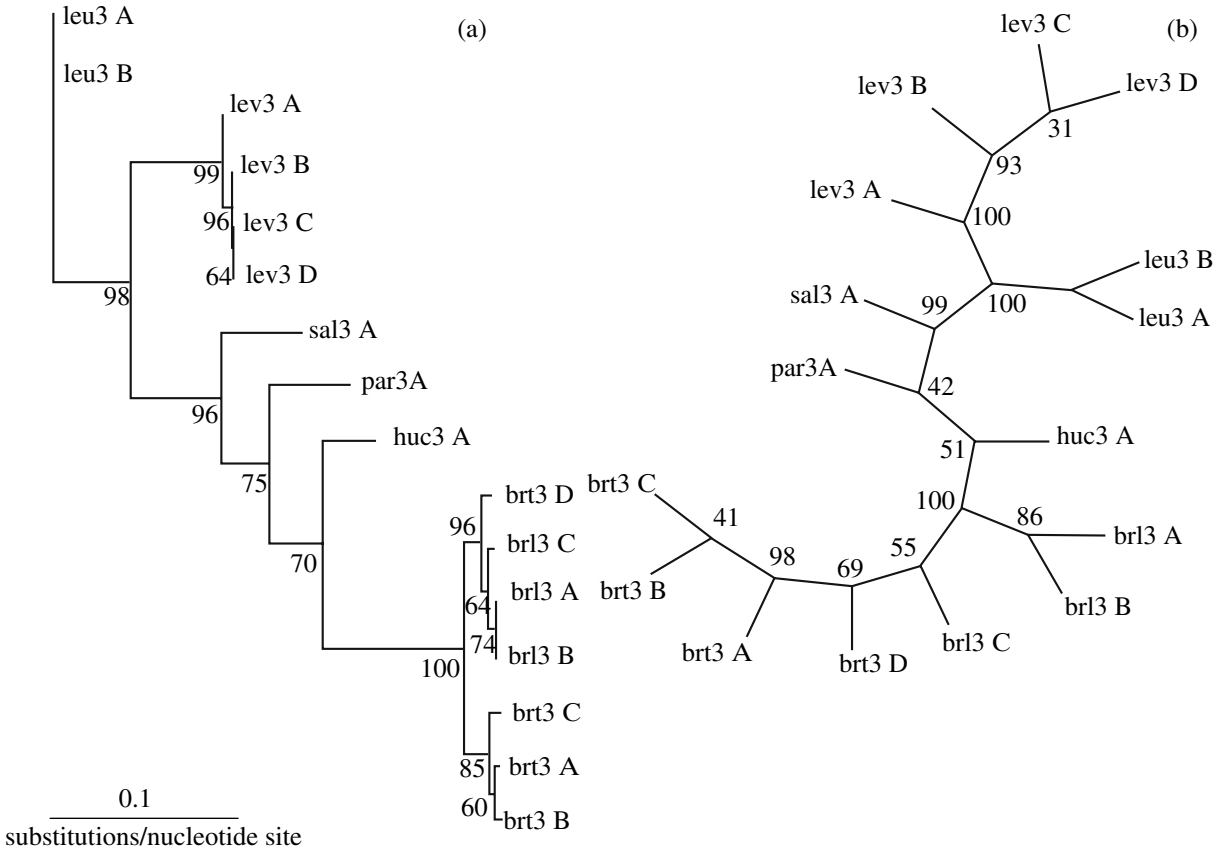

Fig. 3. Comparison of the mtDNA phylogenetic analysis data (the ND3/ND4L/ND4 fragment, 124 phylogenetically informative characters): a, NJ-tree; b, MP-tree. Haplotype designations: *P. perryi*, par3; *H. taimen*, huc3; *B. lenok*, brl3; *B. tumensis*, brt3; *S. salar*, sal3; *S. leucomaensis*, leu3; *S. levanidovi*, lev3. The figures are bootstrap values (in % from 1000 replicates).

tering of the *Salvelinus–Parahucho–Salmo* branches was statistically significant (100%). However, joining of *Parahucho* to *Salvelinus* within this cluster was weakly supported (56%). According to the NJ-tree topology, grouping of *Parahucho* and *Salvelinus* in one cluster was statistically significant (98%), while position of the *Salmo* branch became weakly supported (64%). Phylogenetic analysis performed using maximum parsimony approach with two calculation models did not yield a single most parsimonious tree. A total of 75 trees (tree length, 797 steps) were constructed using the algorithm of the PENNY program, and heuristic search performed with the PAUP package yielded three trees. The trees differed only by the branching orders within the *S. levanidovi* and *Brachymystax* clusters. The MP-tree topology (Fig. 4b) is presented according to the PAUP program, since this tree is most parsimonious (tree length, 791 steps; CI, 0.678; RC, 0.633; RI, 0.934; and HI, 0.322). Topological differences between the three trees were not statistically significant ($P = 1.00$) [30]. Statistical estimate of nonrandom topology of maximum parsimonious MP-tree was high, since all taxon divergence nodes (excluding the *Salmo* branch) showed 100% bootstrap values. The *Salmo* branch occupied the intermediate position between the *Parahucho–Salvelinus* and *Hucho–Brachymystax* clusters in 88% of bootstrap replicates.

Thus, NJ- and MP-tree topologies for the complex of phylogenetically informative characters pooled at the three mtDNA fragments received considerably stronger support than the tree topologies for the individual fragments. Similar results were obtained earlier in analysis of phylogenetic trees generated for the individual genes, as well as for the combined data sets of the mitochondrial and nuclear genes of the salmonid fish [15]. The weak point is the insufficient amount of synapomorphies, i.e., shared characters used as the basis of phylogenetic analysis. Nevertheless, it is noted that some of the mitochondrial genes fairly well determine relationships among the salmonid fish. Among all phylograms generated for 16 mitochondrial genes, Sakhalin taimen was studied only at the *cytb* gene. According to the Bayesian probability tests, *P. perryi* joined the *Salvelinus* cluster earlier than the *Hucho–Brachymystax* group. On the MP-tree, position of the *Parahucho, Salvelinus,* and *Hucho–Brachymystax* branches is not resolved dichotomously [15].

Phylogenetic hypothesis based on the data of RFLP analysis of the mtDNA Cyt*b*/D-loop, ND1/ND2, and ND3/ND4L/ND4 fragments is generally similar to phy-

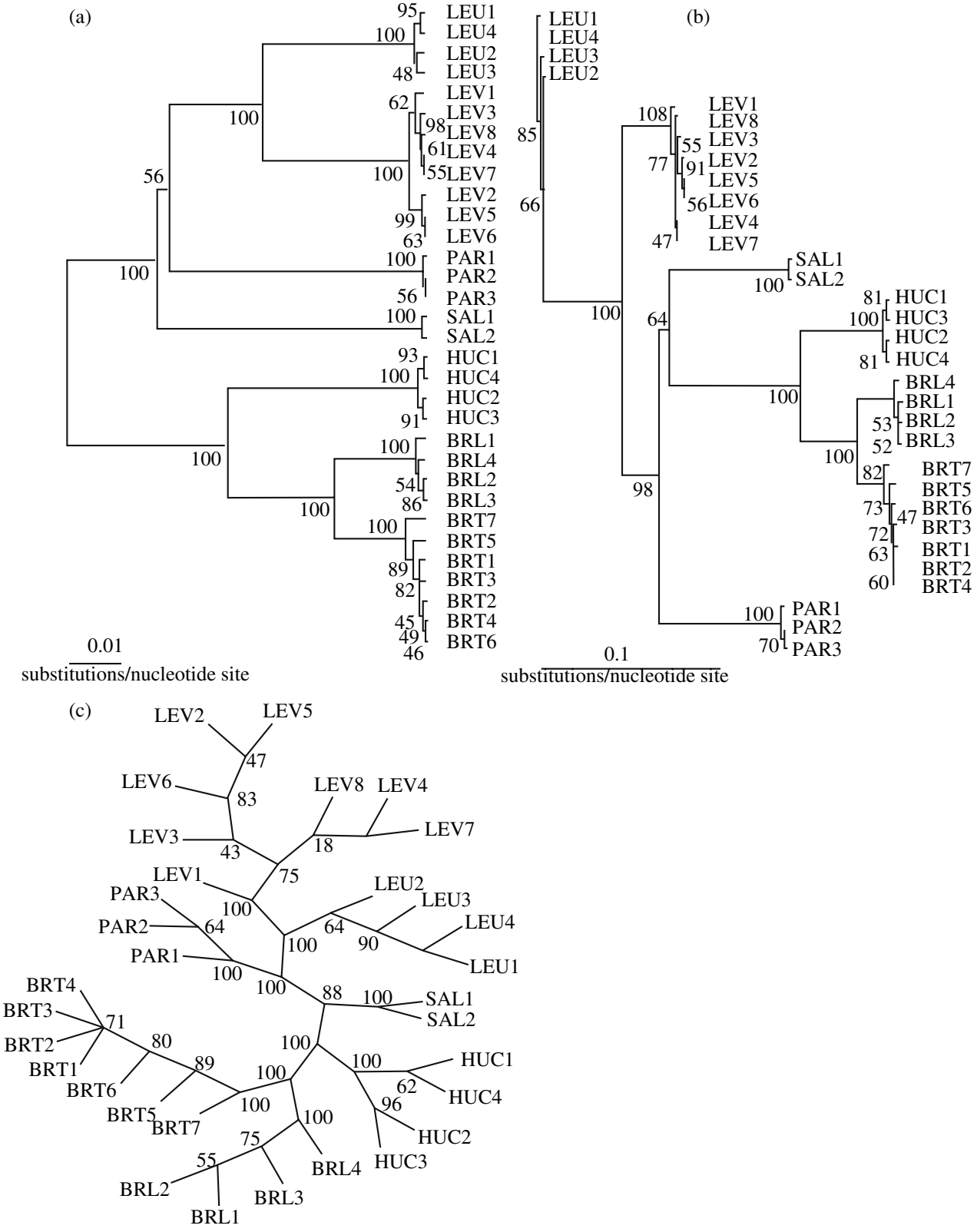

Fig. 4. Comparison of the mtDNA phylogenetic analysis data (the Cyt*b*/D-loop, ND1/ND2, and ND3/ND4L/ND4 fragments): a, UPGMA-tree based on a complex of 574 sites; b, NJ-tree, based on a complex of 536 phylogenetically informative characters; c, MP-tree, based on a complex of 536 phylogenetically informative characters. Haplotype designations: *P. perryi*, PAR; *H. taimen*, HUC; *B. lenok*, BRL; *B. tumensis*, BRT; *S. salar*, SAL; *S. leucomaensis*, LEU; *S. levanidovi*, LEV. The figures are bootstrap values (in % from 1000 replicates).

logenetic schemes inferred from the RFLP analysis of rDNA [10], and sequencing of the intron C of the growth hormone genes *GH-I* and *GH-II* [7, 11]. Validity of the genus *Parahucho* and its substantial divergence from the closely related taxa of *Hucho* and *Brachymystax* is beyond questions. The trichotomy of the *Parahucho*, *Salvelinus, Salmo* branches is explained by the hypothesis, according to which divergence of the genera discussed from the common ancestor occurred very rapidly. The consequence of this process is the small number of synapomorphies determining the relationships among relatied species and the divergence pattern [7].

In conclusion, it should be noted that the phylogenetic scheme obtained is not considered as finite relative the position of *P. perryi*. Inclusion of the outgroup in the analysis will provide the establishment of the trends of evolutionary change, as well as to perform phylogenetic weighting of the characters in relation to the ancestral state. Analysis of other mitochondrial genes is thought to bring additional support to the *Parahucho* branch position. It is evident that data based on analysis of different mtDNA genes are complementary for the purposes of phylogenetic analysis of the taxa. In this case, representative sample consisting of the sites from different genomic regions will provide better understanding of the genome variation than the adjacent sites of a single gene sequences [12, 48–49].

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