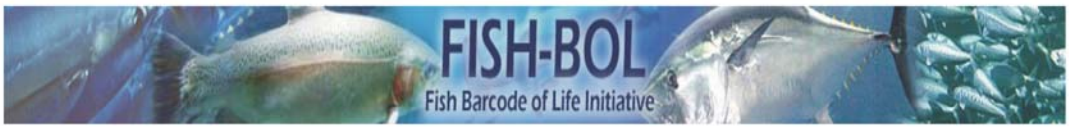
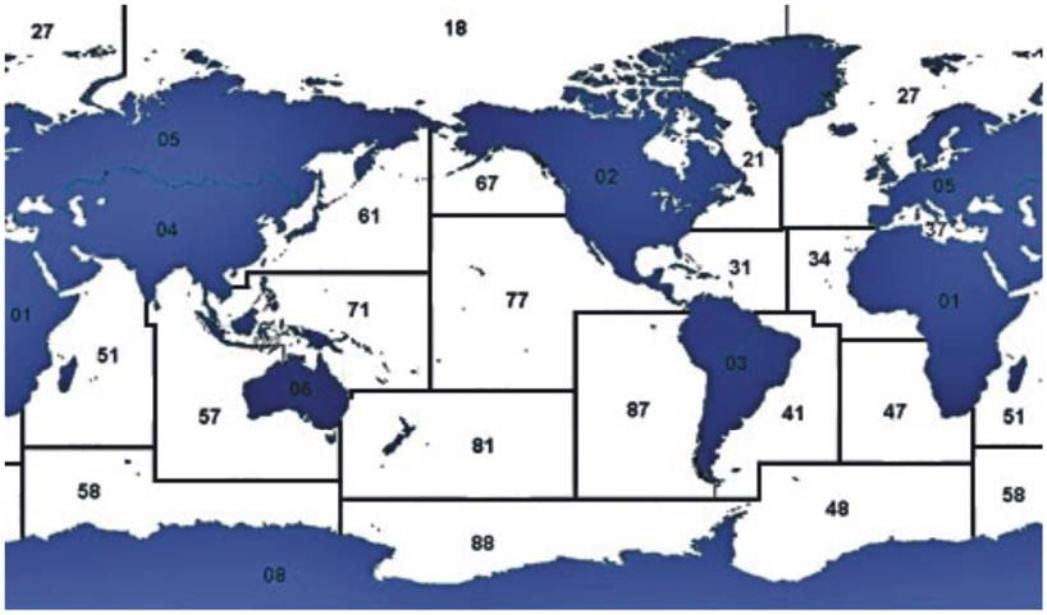


THE INTERNATIONAL WORKSHOP

DNA BARCODING AND MOLECULAR PHYLOGENETICS



Vladivostok, 2008

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DNA BARCODING AND MOLECULAR PHYLOGENETICS

PROGRAM

Held by:

FAR EASTERN BRANCH OF RUSSIAN ACADEMY OF SCIENCES,
A.V. ZHIRMUNSKY INSTITUTE OF MARINE BIOLOGY FEB RAS,
INSTITUTE OF BIOLOGY AND SOIL SCIENCE FEB RAS,
TERRITORIAL ECOLOGICAL FUND OF NAKHODKA CITY,
NAKHODKA CITY ADMINISTRATION,
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Organizing Committee:

Dr. Yuri Ph. Kartavtsev, S.D. & professor (Convener, Russia), Dr. Youn-Ho Lee, professor (Republic of Korea), Dr. Choong-Gon Kim, professor (Republic of Korea), Dr. Kwang-Tsao Shao, professor & director (Taiwan), Dr. Dmitry L. Pitruk, Ph.D. & museum director (Russia), Dr. Sergei V. Shedko, Ph.D. & Project Head (Russia) (Members).

Sunday, Sept. 7

Arrival, accommodation at a hotel in Vladivostok

Monday, Sept. 8

930 - : **Breakfast, downtown excursion at Vladivostok**
1200-1300 : **Lunch**
1400-2300 : **Trip to Vostok MBS, Banquet, Open Fire Party**
2300- : **Spending night at MBS**

Tuesday, Sept. 9

830-900 : **Breakfast and departure to Nakhodka**

10^{00}-13^{00} : **Receipt by the Mayor of Nakhodka City and excursion in Nakhodka**

13^{00}-14^{00} : **Lunch**

14^{00}-17^{00} : **Back trip to Vladivostok, free time**

Wednesday, Sept. 10

8^{30}-9^{30} : **Breakfast**

10^{00}-10^{30} **: Registration & Opening Remarks**

Andrey V. Adrianov, Director, A.V. Zhirmunsky Institute of Marine Biology.
Tatyana A. Chernovalova, Executive director, Nakhodka City Ecological Fund.
Yuri P. Kartavtsev, WSH Convener.

10^{30}-12^{00} : Chair Person – **Prof. Kwang-Tsao Shao**

1. **Yuri Ph. Kartavtsev.** Applicability of sequence diversity at mitochondrial genes on different taxonomic levels in genetics of speciation, phylogenetics and barcoding (40').
2. **Choong-Gon Kim, J. Dageum, E-K. Go, E-J. Choi, Y-C. Lee, K.A. Habib, S-J. Pae, H-K. Yoon, J-W. Chung, S-Y. Hwang, Y-H. Lee.** DNA barcode and oligonucleotide chip for identification of Korean marine organisms (25').
3. **Junichi Imoto, K. Saitoh, Y.P. Kartavtsev, K. Nakamura, M. Miya, M. Nishida, N. Hanzawa.** Phylogenetic analyses of Leuciscinae and related species based on mitochondrial genomes (25').

Lunch Break (60')

13^{00}-17^{00} : Chair Person – **Prof. Choong-Gon Kim**

4. **Kwang-Tsao Shao, K-C. Hsu, P-F. Lee, T-Y. Cheng.** Cryobanking and fish barcod project in Taiwan (25').

5. **Sergey Vladimirovich Shedko, I.L. Miroshnichenko, G.A. Nemkova**. Toward a Systematics and Phylogeography of Eight-Barbel Loaches of the Genus *Lefua* (Cobitoidea: Nemacheilidae) around of the Sea of Japan (25').

6. **Svetlava N. Sharina, Y.Ph. Kartavtsev.** Phylogeny of Far Eastern flatfish species (Pisces, Pleuronectiformes) based on primary sequence of nucleotide at cytochrome oxidase 1 gene (25').

Coffee Break (15')

7. **Evgeniy Stanislavovich Balakirev**, **V.A. Pavlyuchkov, F.J. Ayala.** DNA variation in phenotypically diverse sea urchin *Strongylocentrotus intermedius* and its *Bacteroidetes* symbionts (25').
8. **D.L. Pitruk.** Current state and development of the museum at A.V. Zhirmunsky Institute of Marine Biology (25').

Discussion on Reports (15')

18^{00}-19^{00} : **Dinner, free time**

Thursday, Sept. 11

8^{30}-9^{00} : **Breakfast**
9^{00}-19^{00} : **Free day, Excursions**
18^{00}-19^{00} : **Dinner, free time**

Friday, Sept. 12

8^{30}-9^{30} : **Breakfast**
10^{00}-12^{00} : **Chair Person – Ph.D. Sergei V. Shedko**

8. **Olga Radchenko, I.A. Chereshnev.** Determination of taxonomic rank of genus *Petroschmidtia* (Perciformes: Zoarcidae, Lycodinae) by molecular-genetic analysis (25').
9. **Yuri Ph. Kartavtsev, S.N. Sharina, T. Goto, N. Hanzawa, O.A. Rutenko, A.A.Balanov, D.L. Pitruk.** Investigation of scorpionfish and perch-like species of Russia on cytochrome oxidase 1 gene sequence data with phylogenetic and taxonomic insights (25').
10. **Neonila Polyakova.** COI Sequences in Mugilid DNA Barcoding (25').

Coffee Break (15')

13^{00}-17^{00} : Chair Person - **Prof. Eugeny S. Balakirev**

1. **Kenji Saitoh, Y. Kartavtsev.** Detection and comparative characterization of extinct herring DNA from historical remnants of fishing gear in Hokkaido (25').
2. **Kenji Saitoh, W-J. Chen.** Reducing cloning artifacts for recovery of allelic sequences by T7 endonuclease I cleavage and single re-extension of PCR products -- A benchmark (25').
3. **Se-Jin Pae.** Personal commnication on barcode research experience (5').

General Discussion on Reports (15')

Concluding remarks on WSH meeting: Yuri. Ph. Kartavtsev (15')

1900-2100 : **Dinner & Closing Reception**

<u>Saturday-Sunday, Sept. 13-14</u>

830-900 : **Breakfast**

900-915 : **Departure to Vladivostok and Airport**

ABSTRACTS

DNA VARIATION IN PHENOTYPICALLY DIVERSE SEA URCHIN, *STRONGYLOCENTROTUS INTERMEDIUS* AND ITS *BACTEROIDETES* ENDOSYMBIONTS

E.S. Balakirev[1, 2], V.A. Pavlyuchkov[3], F.J. Ayala[2]

[1] A.V. Zhirmunsky Institute of Marine Biology, Vladivostok 690041, Russia
[2]Department of Ecology and Evolutionary Biology, University of California, Irvine, CA 92697-2525, U.S.A.
[3]Pacific Research Fisheries Centre (TINRO-Centre), Vladivostok, 690600 Russia

Strongylocentrotus intermedius (A. Agassiz 1863) is an economically important sea urchin inhabiting the northwest Pacific region. The Northern Primorye (the Sea of Japan) populations of sea urchin *Strongylocentrotus intermedius* consist of two sympatric morphological forms, "usual" (U) and "grey" (G). The two forms are significantly different in morphology and preferred bathymetric distribution, the G form prevailing in deeper-water settlements. We have investigated nucleotide polymorphism in the fragments of the mitochondrial gene encoding the cytochrome *c* oxidase subunit I (COI) and the nuclear gene encoding bindin (BND) to evaluate the possibility of cryptic species within *S. intermedius*. We have also examined the presence of endosymbiont microorganisms in *S. intermedius* by means of 16S rRNA sequences. The nucleotide sequence divergence between the *S. intermedius* morphological forms is low: 0.74% and 0.73% for the *COI* and *BND* genes, respectively, which is significantly below average intrageneric sequence divergence among *Strongylocentrotus* species. Thus, we have found no evidence of cryptic species within *S. intermedius*. Despite the low level of divergence, the morphological forms have significantly different levels of variability for the *BND* gene, but not for the *COI* gene. The BND protein is known to play a multifunctional role in sea urchin fertilization, mediating the species-specific gamete adhesion and fusion with the egg membrane. Consequently, this gene can be classified as a "speciation gene" (in the sense C.-I. Wu, 2001. J. Evol. Biol. 14, p. 852). Speciation genes may record a phylogenetic history more consistent with the reproductive biology of species than other genes that are not involved in reproductive differentiation. The pattern of molecular evolution is different for the *COI* and *BND* genes in *S. intermedius*. There are two divergent *COI* and *BND* sequence types, which are not congruent and not associated with the sympatric color forms. The level of synonymous variability and divergence is 3.9 and 8.9 times less in *BND* than in *COI*; however the nonsynonymous variability is very similar for both genes but nonsynonymous divergence is 11.6 times less in *COI* than in *BND*. Both genes show significant deviations from neutral expectations. The tree topologies based on the *COI* and *BND* genes are different. 16S rRNA phylogenetic analysis shows that the bacteria endosymbionts of *S. intermedius* belong to the phylum *Bacteroidetes*, but the two morphological forms predominantly harbor highly divergent bacterial lineages, belonging to two different taxonomic classes, *Flavobacteria* in the U form but *Sphingobacteria* in the

G form. We conclude that the U and G forms of *S. intermedius* represent distinct ecomorphological adaptations to contrasting shallow- and deep-water marine environment and might be considered incipient species. The endosymbiotic bacteria likely play an important role in the evolution of morphological and potentially genetic divergence of *S. intermedius*.

PHYLOGENENTIC RELATIONSHIPS OF LEUCISCINAE BASED ON MITOCHONDRIAL GENOME SEQUENCES

J. Imoto[1], K. Saitoh[2], Y. P. Kartavtsev[3,4], K. Nakamura[1], M. Miya[5], M. Nishida[6] and N. Hanzawa[7]

[1] Graduate school of Science and Engineering, Yamagata University, [2] Tohoku Natural Fish Research Institute, [3] A.V. Zhirmunsky Institute Marine Biology, [4]Far Eastern State University, [5]Natural History Museum Institute Chiba, [6]Ocean Research Institute, University Tokyo and [7]Department Biology, Yamagata University

The fishes in the family Cyprinidae that are widely distributed in freshwaters in the world are further classified into 11 subfamilies. One of the subfamily Leuciscinae consists of many species distributed in Northern Hemisphere. The most species in the Leuciscinae are similar morphologically even among different genera. So far, the Leuciscinae species are morphologically divided into two phyletic groups, namely leuciscins for Eurasian plus North American *Notemigonus* species and phoxinins for other North American and Far Eastern species (Cavender and Coburn, 1992). However, due to such morphological similarity, it has been still difficult to infer their phylogenetic relationships and to classify these species. In this study, we conducted analyses based on complete mitochondrial (mt) genome sequences to clarify phylogenetic relationships among European, North American and Far Eastern species in the Leuciscinae.

We determined mitogenome sequences of Leuciscinae fishes and analyzed their phylogeny and estimated divergence times based on their whole mt genomes. The result showed some major clades consisting of Far Eastern, European and North American species. The clade consisting of Far Eastern phoxinins including genera *Tribolodon*, *Pseudaspius* and *Phoxinus* is monophyletic and greatly diverged from the other Leuciscinae species. European leuciscins are located in a monophyletic clade. North American phoxinins including *Phoxinus eos* are divided into some independent clades, whereas *Notemigonus crysoleucas* is located in a sister lineage of European leuciscins. Divergence time estimation showed that Leuciscinae lineages diverged approximately 10–15 million years ago (Mya) and *Tribolodon* species origin 1–5 Mya.

APPLICABILITY OF SEQUENCE DIVERSITY AT MITOCHONDRIAL GENES ON DIFFERENT TAXONOMIC LEVELS IN GENETICS OF SPECIATION, PHYLOGENETICS AND BARCODING

Y.Ph. Kartavtsev

A.V. Zhirmunsky Institute of Marine Biology of the Far Eastern Branch of the Russian Academy of Sciences, Vladivostok 690041, Russia

Algorithms of nucleotide diversity estimates and other measures of genetic divergence at the nucleotide level for two genes *Cyt-b* (cytochrome *b*) and *Co-1* (cytochrome oxidase 1) are analyzed. Based on the theory and algorithms of distance estimates on DNA sequences, as well as on the observed distance values retrieved from literature, it is recommended for realistic tree building to use specific nucleotide substitution model from at least 56 available from Modeltest 3.7 (Posada, Grandall, 1998) or from other software depending on a specific set of nucleotide sequences. Using a database of *p*-distances (Nei, Kumar, 2000, p. 33) and similar measures that were gathered from published sources and sequences derived from GenBank (http://www.ncbi.nlm.nih.gov), genetic divergence of populations (1) and taxa of different rank, such as subspecies or/and sibling species (2), species within a genus (3), species from different genera within a family (4), and species from separate families within an order (5) have been compared.

Empirical data for approximately 25 000 vertebrate and invertebrate species demonstrate that the data series are realistic and interpretable when *p*-distance and its various derivates are used. The focus was made on vertebrates and fish species in particular and the newest dataset obtained in the frame of FishBOL (http://www.fishbol). Distance data revealed various and increasing levels of genetic divergence of the sequences of genes *Cyt-b* and *Co-1* in five groups compared. Mean not weighted scores of *p*-distances for five groups are equals to: *Cyt-b* (1) 1.55±0.56, (2) 5.52±1.34, (3) 10.69±1.34, (4) 18.51±2.09 (5) 19.81±0.14 and *Co-1* (1) 0.55±0.19, (2) 4.91±0.83, (3) 9.66±0.72, (4) 14.69±1.02, (5) 18.15±0.18. The estimates show good correspondence with the former data (Johns, Avise, 1998; Hebert et al., 2004; Ward et al., 2005; Kartavtsev, Lee, 2006). This testifies to the applicability of *p*-distance for most intraspecies and interspecies comparisons of genetic divergence up to the order level by two genes compared. However, as seen from the numbers above for the family-and-order level there is a sign of a certain inflation, probably caused by a homoplasy effect. Diversity among other genes is also compared for different taxa levels and showed a similar pattern.

Differences in divergence between the genes themselves at the five hierarchical levels were also found. This conforms to the ample evidence showing different and nonuniform evolution rates of these and other genes and their various regions. The results of the analysis of the nucleotide as well as allozyme divergence within species and higher taxa of animals, firstly, are in a good agreement with the known results and showed the stability of a general trend; secondly, these evidences suggest that in animals, phyletic evolution is

likely to prevail at the molecular level, and speciation mainly corresponds to the geographic model (type D1). The prevalence of the D1 speciation mode does not mean that the other modes are absent. There are at least seven various modes of speciation (Templeton, 1981; Kartavtsev et al., 2002, Kartavtsev, Lee, 2006). How can we recognize them formally is a key question to establish quantifiable genetic model (theory) of speciation. An approach is suggested that allows to step forward in this direction following own and literature developments (Templeton, 1981; 1998; DeQueiros, 1998; Harrison, 1998; Howard, 1998; Wu, Hollocher, 1998; Kartavtsev et al., 2002, Kartavtsev, Lee, 2006). In the approach developed two main points are important: (1) a belief that because of the complexity of species criteria, the distance data alone may give only a part of necessary information for the discrimination among speciation modes, and (2) a precise classification of speciation modes, based on certain formal criteria but tested experimentally with a set of measurable descriptors, is a perspective way for solving such a complicated matter as speciation.

Research was supported by the Russian Foundation for Fundamental Research grants **#07-04-00186, #08-04-91200** and **the** Far Eastern Branch of the Russian Academy of Sciences grant **#08-3B-06-031**.

INVESTIGATION OF SCORPIONFISH AND PERCH-LIKE SPECIES OF RUSSIA ON CYTOCHROME OXIDASE 1 GENE SEQUENCE DATA WITH PHYLOGENETIC AND TAXONOMIC INSIGHTS

Y.Ph. Kartavtsev[1], S.N. Sharina[1], T. Goto[2], N. Hanzawa[2], O.A. Rutenko[3], A.A.Balanov[1], D.L. Pitruk[1]

[1]A.V. Zhirmunsky Institute of Marine Biology, Vladivostok 690041, Russia, [2]Department of Biology, Faculty of Science, Yamagata University, Yamagata 990-8560, Japan; [3]Far Eastern State University, 690060, Vladivostok, Russia

Mitochondrial DNA (mtDNA) at cytochrome oxidase 1 (*Co-1*) gene region was sequenced for 7 scorpionfish and 9 perch-like fish species (in total, 16 and 11 sequences of at least 552 bp) from the Far East of Russia and compared with other sequences of Scorpaeniformes and Perciformes comprising altogether 29 vs 20 scorpionfish and perch-like fish sequences and two outgroup sequences (Cypriniformes). The analysis of the protein-coding *Co-1* gene revealed statistically substantiated bias in the (T+C) : (A+G) content, proving basic findings. The average scores of *p*-distances for different scales of the evolutionary history at *Co-1* gene revealed a clear pattern of increased nucleotide diversity at four different levels: (1) intraspecies, (2) intragenus, (3) intrafamily, and (4) intraorder. The scores of average *p*-distances for the compared scorpionfish groups were: (1) 1.00±0.20%, (2) 3.80±0.20%, (3) 12.40±1.20%, and (4) 18.00±0.38%, and for perch-like fishes were: (1) 0.11±0.04%, (2) 1.87±0.68%, (3) 12.67±0.28%, and (4) 16.52±0.10%, respectively (mean ± SE). These data support the concept that speciation in the orders Scorpaeniformes and Perciformes, in most cases, follows a geographic mode through

accumulation of numerous small genetic changes over a long period of time. However, intraspecies diversity was surprisingly high among scorpionfish. Phylogenetic trees for 29 sequences of scorpionfish and 2 other fishes belonging to ray-finned fishes (Actinopterigii) were developed using *Co-1* gene and four different analytical approaches: Bayesian (BA), maximum likelihood (ML), neighbour-joining (NJ), and maximum parsimony (MP). The analysis revealed a monophyletic origin for the representatives of Cottidae, which is the principal scorpionfish family investigated (100, 96, 98% support level in our BA, MP, and NJ analyses). Similarly, the monophyletic origin of up to the three compared scorpionfish genera was supported by molecular phylogenetic data. The analysis revealed a monophyletic origin for the representatives of Stichaeidae, which is the principal Percoids family investigated (100, 84, 86% support level in our BA, ML, and NJ analyses). According to the current and literary data, the monophyletic origin up to the four compared perch-like fish families was supported by molecular phylogenetic data: Carangidae, Sparidae, Zoarcidae, Stichaeidae. However, more numerous taxa representation is necessary for precise conclusion on this point. Species identification on individual basis (barcoding tagging) was high among representatives of both orders. A few taxonomic complications arose during the analysis and they are discussed here in.

DNA BARCODE AND OLIGONUCLEOTIDE CHIP FOR IDENTIFICATION OF KOREAN MARINE ORGANISMS

C.-G. Kim[1], D. Jeong[1], G.-E. Kim[1], E.-J. Choi[1], Y.-C. Lee[1], K.A. Habib[1], H.-S. Park[1], S.-J. Pae[1], H.-K. Yoon[2], J.-W. Chung[2], S.-Y. Hwang[2], Y.-H. Lee[1]

[1]Korea Ocean Research and Development Institute (KORDI), P.O. Box 29, Ansan 425-600, Seoul KOREA (e-mail: kimcg@kordi.re.kr)

[2]GenoCheck Co., LTD., Gyeonggi-Do, KOREA

We analyzed more than 250 species of COI barcode of the Korean marine organisms which are including fish, sea urchin, sea cucumber, sponge, and mollusca. The COI barcode results showed that a significant differentiation between each species using this study except the sponge species.

As a rapid genetic species identification, some of these organism, we developed a DNA microarrays with oligonucleotide probes based on the DNA barcoding technology. Oligonucleotide probes have been designed from COI gene sequences. To test the usefulness of the DNA chip, fragments of the COI gene were amplified from Korean marine organisms and hybridized with the oligonucleotid probes. Distinct species specific hybridization patterns appeared on the DNA chip dependent on the species. In contrast with conventional DNA sequencing, microarray hybridizations require only one PCR and purification step, allowing faster and easier handling. These results show that DNA chip method are a feasible technology for species identification in marine organisms.

COI SEQUENCES IN MUGILID DNA BARCODING

N.E. Polyakova, A.V. Semina, V.A. Brykov
A.V. Zhirmunsky Institute of Marine Biology,
Far Eastern Branch of Russian Academy of Sciences

In the present study we have analyzed COI sequences of nine Mugilid species: *Mugil cephalus* (Sea of Japan and Azov, Mediterranean, Taiwan), *Liza saliens* (Mediterranean), *L. aurata* (Sea of Azov, Mediterranean), *L. ramado* (Mediterranean), *L. macrolepis* (Taiwan), *L. subviridis* (Taiwan), *Chelon labrosus* (Mediterranean), *C. haematocheilus* (Sea of Japan, Taiwan, and introduced to the Sea of Azov), and *Valamugil cunnesius* (Taiwan). The data obtained showed that information based on COI sequences was diagnostic not only for species-level identification but also for recognition of intraspecific units, e.g. allopatric populations of circumtropical *M. cephalus*, whose pronounced population-genetic structure is well-known, or even native and acclimatized specimens of *C. haematocheilus*. Despite the lack of informative sites and saturation effect, topologies of phylogeny correspond to our previous results based on PCR-RFLPs of extended mtDNA segments. The results of COI analysis has contributed to the long debates over monophyly of the *Liza* genus. It confirmed that *Liza* species were not monophyletic exclusively of *C. labrosus*, which supports the idea of *Liza* and *Chelon* unnatural subdivision into two genera and recommendation of their synonymization with the priority of *Chelon*. Thus, all the *Liza* species should be ascribed to the genus of *Chelon*, hence solving existing disagreement in the taxonomic status of Far Eastern mullet, *C. haematocheilus*.

DETERMINATION OF TAXONOMIC RANK OF GENUS *PETROSCHMIDTIA* (PERCIFORMES: ZOARCIDAE, LYCODINAE) BY MOLECULAR-GENETIC ANALYSIS

O.A. Radchenko, I.A. Chereshnev
Institute of Biological Problems of the North, Russian Academy of Sciences

Petroschmidtia albonotata Taranetz et Andriashev, 1934 from the family Zoarcidae was described by the set of individuals, caught at a depth of 202-220 m in the north-eastern part of the Sea of Okhotsk (Taranetz, Andriyashev, 1934). The authors suggest *Petroschmidtia* to be close to genus *Lycodes* and relative genera of subfamily Lycodinae, but it also has a series of distinctive morphological characteristics. However, Anderson M. E. came to conclusion, that the level of morphological distinctions of *Petroschmidtia* from

other genera Lycodinae is insufficient to define it as a separate genus. Thus, *Petroschmidtia* should be considered not higher, than subgenus rank of *Lycodes* genus.

To identify the taxonomic status of genus *Petroschmidtia*, which is endemic to the Sea of Okhotsk and the Sea of Japan, the first molecular-genetic analysis of the species from this genus and of two related genera, *Lycodes* and *Bothrocarina,* has been carried out. Nucleotide sequences of COI, cytochrome b, 16S rRNA genes of mitochondrial DNA (mtDNA), in total 2043 nucleotide pairs long, have been determined. The mean of genetic divergence between mitochondrial DNA sequences of *Petroschmidtia* and *Lycodes* was found to be 7.0%, between *Lycodes* and *Bothrocarina* – 6.7%, between *Bothrocarina* and *Petroschmidtia* – 8.2%. By phylogenetic analysis of mtDNA nucleotide sequences we have defined phylogenetic trees, showing species relationships of subfamily Lycodinae. The basement of the tree is formed by haplotype of the out group taxon, *Pholidapus dybowskii* Steindachner, 1880 (family Stichaeidae, suborder Zoarcoidei). Then stand out *Hadropareia middendorffii* Schmidt, 1904 (subfamily Gymnelinae, family Zoarcidae) and macrocluster of subfamily Lycodinae. The latter is subdivided into three clusters, corresponding to genera *Petroschmidtia*, *Bothrocarina* and *Lycodes*. The cluster of MtDNA for *Lycodes* is located closer to *Bothrocarina*, than to *Petroschmidtia*. The nucleotide divergence values of mtDNA sequences for Lycodinae subfamily and topology of phylogenetic trees showed, that level of genetic distinctions of *Petroschmidtia* from *Lycodes* and *Bothrocarina* genera exceeds that between two last genera. This indicates *Petroschmidtia* to be a separate genus of Lycodinae subfamily.

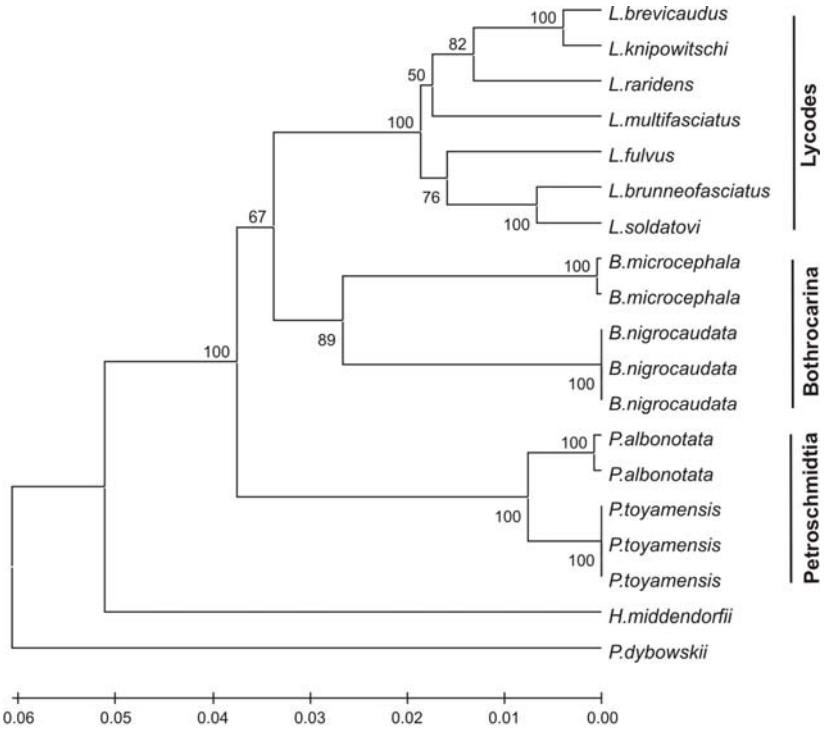

Fig. UPGMA phylogenetic consensus tree derived from analysis of nucleotide sequences of the mtDNA of the subfamily Lycodinae species. Numbers on branches indicate consensus analysis values. Numbers on scale indicate genetic distances

DETECTION AND COMPARATIVE CHARACTERIZATION OF EXTINCT HERRING DNA FROM HISTORICAL REMNANTS OF FISHING GEAR IN HOKKAIDO

K. Saitoh[1], Y. Kartavtsev[2]

[1]Tohoku National Fisheries Research Institute, Fisheries Research Agency, [2]A.V. Zhirmunsky Institute of Marine Biology, Far East Branch, Russian Academy of Sciences

The Hokkaido-Sakhalin stock of herring had once been abundant on the Sea of Japan coast of Hokkaido some 100 years ago. The herring fishery collapsed about 50 years ago, and this stock has been extinct from Japanese coast for these fifty years. Genetic analysis of this stock helps understanding of population structure and long-term stock fluctuation of Pacific herring. Several historical fishing facilities remain in Hokkaido, and herring scales attach onto fishing gear kept in these facilities or history museums. In this study we sequenced 302bp of mitochondrial control region from these herring scales and compared them with the present-days herring stocks from Japan, Russia and Alaska. Scales were come from 15 fishing gears from six localities. Among 270 scales examined, herring mtDNA fragments were amplified from 17. DNA fragments from some scales showed a number of consensus sequences, as well as some putative PCR errors, upon cloning of the PCR-amplified fragments. On the other hand, two or more scales rarely harbor the same sequence. Therefore, each scale was mostly from independent individual, and some scales keep body material from a number of individuals through fishery landing and handling. Thus, DNA sequences from scales represent the herring population to some extent. We will compare the sequences with those from present-days herrings to discuss retrospective geographic population structure of Pacific herring.

REDUCING CLONING ARTIFACTS FOR RECOVERY OF ALLELIC SEQUENCES BY T7 ENDONUCLEASE I CLEAVAGE AND SINGLE RE-EXTENSION OF PCR PRODUCTS -- A BENCHMARK

K. Saitoh[1], W.-J. Chen[2]

[1]Tohoku National Fisheries Research Institute, Fisheries Research Agency, [2]Saint Louis University, Department of Biology

Occurrence of chimeric sequences and related artifacts in PCR cloning procedures gives us risks of over-estimation of haplotypes or alleles. Recombination among haplotypes occurs through template switching during PCR cycles or through random repair of mismatch sites on heteroduplex DNA by the host cell. To eliminate the chimeric

cloning artifacts, we tested two alternative protocols using T7 endonuclease I cleavage of mismatch sites and re-extension of nascent strands. Though T7 endonuclease I effectively eliminated chimeric clones in some cases, it produced many short fragments. Protocol with single re-extension of PCR products successfully recovered non-recombinant clones with fewer short fragments. In spite of the improvement of allelic recovery through these two protocols, there were still a few recombinants remained in both reaction mixtures, and thus interpretation of the results for haplotype diversity in a PCR-amplified DNA population should be cautionary. Because re-extension in a diluted reaction mixture is quick, inexpensive and effective, it is advisable to use this procedure for recovery of chromosomal alleles with PCR cloning.

CRYOBANKING AND FISH BARCODE PROJECT IN TAIWAN

K-T. Shao[1], K-C. Hsu[1], H-L. Ko[1], H-T. Shy[1], P-F. Lee, T-Y. Cheng[1], Y-C. Liao[1]
[1]*Biodiversity Research Center, Academia Sinica*

Although only the Academia Sinica and National Sun Yat-Sen University joined the CBOL in 2004, a four-year "Cryobanking of wild animal genetic materials from Taiwan" project was carried out by five institutions in 2005 with the support of the Council of Agriculture. So far, a total number of 8,400 individuals and 2,140 species, including 1120 species of fish, 115 amphibians and reptiles, 100 birds, 55 mammals and 750 insects and invertebrates have been deposited at various museums. Moreover, all spared specimens will be deposited at the Livestock Research Institute, COA. All specimen data now can be browsed from the Taiwan cryobanking database (http://cryobank.sinica.edu.tw).

Various barcode projects have been conducted in Taiwan, including fungi, plants, seaweeds, some insects, crustaceans, terrestrial mollusks, fishes, amphibians, reptiles, birds and terrestrial and marine mammals. Fish barcode project is mainly carried out by Academia Sinica including establish Fish-BOL database for all economic fish species in Taiwan and many projects related to molecular identification for fish eggs and larval fishes and its applications on taxonomy, phylogeny and ecological studies. For example, 11 species including one new species of gobies could be identified from 24 morphotypes collected at Lanyang Estuary. The classification problems of *Trichurus* (Cutlassfishes) could be solved based on the Cyt b, 16 S rRNA and COI. Based on the preliminary surveys of our 500~1000 fish species samples, we noticed that the cyt b gene, in addition able to identify populations, can identify species with the same power as the COI gene can. So, we suggest that in the future we should establish a cyt b database too.

PHYLOGENY OF FAR EASTERN FLATFISH SPECIES (PISCES, PLEURONECTIFORMES) BASED ON PRIMARY SEQUENCE OF NUCLEOTIDES AT CYTOCHROME OXIDASE 1 GENE

S.N. Sharina, Y.Ph. Kartavtsev

A.V. Zhirmunsky Institute of Marine Biology of Far Eastern Branch of Russian Academy of Sciences, Vladivostok 690041, Russia

Nowadays one of the most popular for molecular phylogenetics is cytochrome oxidase 1 (*Co-1*) gene sequences, which is usually utilized for species-and-family level analysis (Hebert et al, 2002). Abundant data demonstrate that sequence data for *Co-1* gene are usually suitable for species discrimination, and very helpful in phylogenetic reconstructions, including numerous fish taxa (Ratnasingham, Hebert, 2007).

The primary nucleotide sequences at *Co-1* gene of mitochondrial DNA (mtDNA) were taken for investigation. Total, including GenBank (NCBI) data, 26 sequences of flatfish and 2 sequences of Perciformes as outgroup were analyzed.

The analysis of base composition revealed the following nucleotide content: T – 28.4%, C – 27.2%, A – 25. 3%, G – 19.1% (averages), which deviated from 1:1:1:1 ratio and that is well agreed with earlier data for protein-coding genes. There is no one sequence for which the nucleotide composition deviated significantly from the average. Therefore, we can expect stable outcome from the phylogenetic analyses in specified species group.

On the first step for comparison of a sequence divergence the *p*-distance scores were calculated on pair-wise basis for different taxonomic groups of the representatives of flounders. These calculations provided rough representation on nucleotide diversity at the four phylogenetic levels: (1) intraspecies, (2) intragenus, (3) intrafamily and (4) intraorder. Scores of mean *p*-distances revealed a clear pattern of increased values for the four categories of comparison (1) 0.09$\pm$0.06%, (2) 0.97$\pm$1.87%, (3) 11.98$\pm$0.63%, (4) 20.09$\pm$0.17%, correspondingly (mean$\pm$SE) (Sharina et al, 2007). The *p*-distance data allowed the conclusion that in the order Pleuronectiformes speciation in most cases followed a geographic mode. Such increase of genetic diversity level with taxa rank was obtained earlier for bulk of species groups (Johns, Avise, 1998, Hebert et al, 2003, Ward et al, 2005, Kartavtsev et al, 2007 a,b) and wider list of taxa categories (Johns, Avise, 1998, Kartavtsev, Lee, 2006) supporting our notion.

For visualization of phylogenetic relationships among flounder representatives several kinds of phylogenetic trees were created. We based here on two approaches: Bayesian (BA) and neighbor joining (NJ). These two trees gave principally similar topologies, proving the monophyly of representatives of 4 investigated families of flatfish order Pleuronectiformes. Well genetically distinct are the majority of genera and species of flatfish; it means that their taxonomic position is unambiguous. Individuals, which belong to the same species, are also clustered in the nodes with fine support. Thus, from data presented we can conclude that the species diagnostics based on *Co-1* gene is efficient because of low intraspecies and high interspecies variability of these markers. Also, the

molecular-phylogenetic reconstructions, which were obtained earlier at *16S rRNA* (Cooper and Chapleau, 1998) and *Cyt-b* (Kartavtsev et al, 2007 b) genes well confirm our even partial sequence data at *Co-1* gene. However, some complications are available and there will be included in the discussion.

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TOWARD A SYSTEMATICS AND PHYLOGEOGRAPHY OF EIGHT-BARBEL LOACHES OF THE GENUS *LEFUA* (COBITOIDEA: NEMACHEILIDAE) AROUND OF THE SEA OF JAPAN

S.V. Shedko, I.L. Miroshnichenko, G.A. Nemkova
Institute of Biology and Soil Science, Far East Branch of Russian Academy of Sciences, Vladivostok, 690022, Russia; e-mail: shedko@ibss.dvo.ru

Eight-barbel loaches of the genus *Lefua* Herzenstein, 1888 are small fresh-water fishes, inhabiting the rivers and lakes of the eastern part of Asia. It is accepted (Berg, 1948; Zhu, 1989; Nakabo, 2002) that the genus consists of four species. One of these species, *L. costata* (Kessler, 1876), is widely distributed on the continent (from the Amur River basin to the Chinese province Shandong in the south). The other species are found on the islands: *L. echigonia* Jordan et Richardson, 1907, on Honshu Island; the taxonomically not yet legalized *Lefua* sp., in the southwest of Honshu Island and on Shikoky Island; *L. nikkonis* (Jordan et Flower, 1903), on Hokkaido Island, northern part of Honshu Island (Prefecture Aomori), and the southern part of Sakhalin Island. Recent studies of genetic differentiation of *Lefua* from the islands of Japanese Archipelago (Saka et al., 2003; Sakai et al., 2003; Mihara et al., 2005) generally confirmed the accepted structure of the genus. According to these studies, *L. nikkonis* was close to *L. constata* (the latter species was represented by samples from the south of the Korea Penninsula), while *L. echigonia* and *Lefua* sp. were well-differentiated lineages. Recently, it was demonstrated (Naseka, Bogutskaya, 2004) that eight-barbel loaches from the south of Primorskii krai were not morphologically identical to typical *L. costata* from the northeast of China, and should be considered as an distinct species, *Lefua pleskei* (Herzenstein, 1887). For these reasons, in the present study genetic divergence and phylogenetic position of eight-barbel loaches from different localities of Primorye (including the type locality of *L. pleskei*, Ilistaya River) among the other groups of the genus *Lefua* was evaluated based on sequence analysis of the mtDNA control region and cytochrome b gene sequences (about 2070 bp a total).

Comparative analysis of own sequence data for eight-barbel loaches from eight localities in the Amur River basin (4), the Sea of Japan (4) and the GeneBank/NCBI data for the eight-barbel loaches from the other regions of the East Asia revealed that eight-barbel loaches from Primorskii krai water basins have a specific group of mtDNA

haplotypes. This finding is considered as supporting the species status of *L. pleskei*. Genetic distances within *L. pleskei* are small (on average 0.4%) and close to those within *L. nikkonis* (on average 0.5%). The distances between this species pair are the least (on average 2.2%) among all other pair comparisons. In MP, ML, and Bayesian trees, *L. pleskei* and *L. nikkonis* haplotypes formed a common clade with high statistical support. In all tree variants, *L. costata* mtDNA haplotypes were located from outside of forementioned clade. A clade included of highly diverged lineages of *Lefua* sp. and *L. echigonia* haplotypes occupied basal position. The mtDNA haplotypes of *L. pleskei* and *L. costata* from the Amur River basin were evolutionary young and derived from the haplotypes found in these species from the Sea of Japan (*L. pleskei*) or the Yellow Sea (*L. costata*) basins. It is thereby suggested that both species rather recently migrated into the Amur River system. According to the molecular dating, basal diversification of the eight-barbel loach lineages probably took place at the end of middle Miocene (about 11 to 12 Myr ago), while divergence of *L. pleskei* and *L. nikkonis* ancestral forms probably occurred approximately, 5 Myr ago. Since all main lineages of eight-barbel loaches were found in the Sea of Japan basin (continental coastline and the islands), the divergence order and dispersal patterns of the *Lefua* species might have been largely determined by the geological development of this water body and the adjacent territories.

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