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The phylogeny based on the intron of ATP synthase α-subunit gene reveals a substrate role in the origin of the gastropod species flock from Lake Baikal

Kovalenkova M.V.[1]*, Bukin Yu.S.[1, 2], Sitnikova T.Ya.[1], Sherbakov D.Yu.[1, 2]

[1] *Limnological Institute, Siberian Branch of the Russian Academy of Sciences, Ulan-Batorskaya Str., 3, Irkutsk, 664033, Russia*
[2] *Irkutsk State University, Karl Marx Str., 1, Irkutsk, 664003, Russia*

ABSTRACT. A nuclear marker, an intron of ATP synthase α-subunit gene, used to determine phylogenetic relationships between 22 species of the family Baicaliidae (Caenogastropoda: Rissooidea) indicated two genetic groups of these gastropods in Lake Baikal. The obtained groups partially coincide with the clusters revealed by the mitochondrial COI gene and do not fully coincide with the modern taxonomy of the family. Thirteen species of the first group inhabit different sediments (silty sand, sand, stones, and rocks), eight out of the nine species from the second group are psammobionts. Both genetic groups include eurybathic and stenobathic species widely distributed in the North, Middle and Southern Basins of the lake, as well as species, whose habitats are limited to one or two basins. Phylogenetic analysis suggests that the main way of baicaliids speciation was the repeated occupation of the same habitats by different ancestral forms. Sculpture of the shells (ribs, carina and/or periostracal hairs/plates) also most likely appeared independently in each group due to similar adaptation.

Keywords: substrate preferences, sand-dwelling, Baicaliidae

1. Introduction

The processes generating the unique biodiversity in ancient lakes remain not quite clear, giving rise to numerous hypotheses (Cristescu et al., 2010). In the understanding of the driving forces of evolution, of particular interest are the processes leading to the rapid emergence of a large number of closely related species (species flocks) (Brooks, 1950). In ancient lakes, periods of intense speciation is normally associated with the consequences of some rapid catastrophic changes that caused the formation of a large number of new and/or unpopulated ecological niches (Martens, 1997; Sherbakov, 1999; Kroll et al., 2012; Miura et al., 2019). During adaptive radiation morphological, environmental and behavioral characteristics of closely related organisms can change very quickly (Martin and Wainwright, 2011).

The endemic gastropods of the family Baicaliidae (Fischer 1885) include more than 40 species belonging to 8 genera and it is the largest species flock among gastropods from ancient lakes. Family or subfamily rank of this group has been considered (Wilke, 2004; Wilke et al., 2013; Vinarski and Kantor, 2016). Baicaliids are found on all types of sediments at the depth from 1.5 to 250 m, most species were collected at the depths down to 50 m (Kozhov, 1936; Sitnikova et al., 2004). Some species widely distributed in the lake; the habitat of a few species is limited to one-two basins or a single geographical part (northern or southern) of the lake. There are pairs of species (or subspecies) of similar shell morphology and occupying similar habitats but in different lake basins (Kozhov, 1936; Sitnikova, 2006). Sympatric distributed species differ in time of reproduction (Sitnikova et al., 2001), reproductive behavior (Sitnikova, 2004) or trophic preferences (Sitnikova et al., 2012). Previously diversification within macro- and microhabitats was proposed as the main way of the speciation of the Baikal endemic gastropods (Starobogatov and Sitnikova, 1990).

The relative evolutionary youth of contemporary baicaliids and rapid evolution significantly complicate the study of their phylogenetic relationships (Zubakov et al., 1997). Analysis of polymorphism of cytochrome c oxidase gene (COI) fragment in 25 species indicated two genetic baicaliid clusters that do not fully correspond to the taxonomy of the family based on morphological characteristics (Zubakov, 1999). The morphological variability of the shells in the members of the family suggests the presence of transitional forms and interspecific hybrids (Kozhov, 1936). Furthermore, the intraspecific genetic variation of the COI gene in some species exceeds interspecific differences (Peretolchina et al., 2007). The intron of the nuclear ATP synthase

*Corresponding author.
E-mail address: mkovalenkova@gmail.com (M.V. Kovalenkova)

α-subunit gene served in our study as a variable and neutral marker for the evolution of baicaliids (Kovalenkova et al., 2015) because the mitochondrial genome may undergo frequent events of different types of genetic selection being a single segregation unit due to lack of recombination (Chong et al., 2016; Sun et al., 2017).

This study aims to evaluate the intron variability of the ATP synthase α-subunit gene in gastropods of the family Baicaliidae as well as the relationship of genetic polymorphism with some known environmental and morphological characteristics of species.

2. Materials and methods

Gastropods belonging to all baicaliid genera were collected in 2006–2017 with dredging and scuba diving from 22 littoral and sublittoral sites (5-40 m) (Table).

Shells were photographed before dissecting. We isolated genomic DNA from foot tissues using a CTAB procedure described by Doyle and Dikson excluding isoamyl alcohol adding (1987).

Intron-containing partial fragment of ATP synthase α-subunit gene was obtained using primers universal for coelomate animals (Jarman et al., 2002). Thermocycling conditions were as follows: 94 °C – 2 min, 35 cycles: 94 °C – 20 s, 54 °C – 1 min, 72 °C – 1 min (with a final elongation step of 3 min). PCR products of the intron were separated on 1% agarose gel and purified using the freeze-squeeze method (Tautz and Renz, 1983). Then the amplification products were sequenced at JSC Sintol (Moscow). GenBank accession numbers are shown in Table.

Sequenograms were edited and aligned with Geneious v. 9.0.5 (Kearse et al., 2012). Double peaks were named according to IUPAC nomenclature with Heterozygotes plugin (Geneious) using a threshold of 50% peak height. Intron alleles for heterozygous specimens were twice identified using the PHASE algorithm in DnaSP v. 5.10.01 (Librado and Rozas, 2009). Reconstruction results were the same. The best-fit model of sequence evolution was inferred based on the Bayesian information criterion with IQ-tree v. 1.6.10 (Nguyen et al., 2015).

Maximum Likelihood (ML) tree from unique alleles of the intron was constructed with IQ-tree v. 1.6.10. In order to assess the robustness of the tree topology, bootstrap node support values were estimated from 100 pseudoreplicates. ML tree have been inferred without an outgroup because of the lack of the sequence information for the ATP synthase α-subunit gene intron for any sister taxa.

Medians of paired interspecific genetic distances were estimated with the F81 + I model (Felsenstein, 1981) in the “ape” package R (Paradis et al., 2004). The statistical parsimony network (Templeton et al., 1992) was inferred with the fixed connection limit set to 97% in the “pegas” package R (Paradis, 2010).

Ecological characters (minimum and maximum habitat depths limits, substrate preferences, habitat area) and teleoconch sculpture characters were assigned according to Kozhov (1936) and Sitnikova (2004) (Table).

The relationship of environmental and morphological characteristics with genetic differentiation was assessed with non-metric multidimensional scaling (NMDS) using the matrix of median pairwise interspecific distances as source data. Qualitative and quantitative environmental and morphological characteristics served as grouping factors of species distribution in the space of NMDS coordinates to calculate the ratios of intra-factor and inter-factor variations for interspecific median distances, R2 ($R2 = 1 - ssw/sst$, where ssw and sst are within-group and total sums of squares). The greater the R2 value for the studied factor, the greater its influence on the degree of genetic differentiation between species. The reliability of the R2 value was calculated using 3000 repetitions of permutation test (Phipson and Smyth, 2010) and adjusted by the Benjamini and Hochberg method for multiple comparisons (Benjamini and Hochberg, 1995). If $P < 0.05$ then R2 significantly differs from zero, and the influence of the factor on the interspecific genetic differentiation is considered reliable. The calculations were carried out using the “vegan” package R according to the recommendations of Oksanen (2015).

3. Results

We obtained the nucleotide sequences of the intron of the ATP synthase α subunit gene for 55 specimens belonging to 22 species (1-6 specimens per species). The intron length varied from 466 to 523 bp in different species due to short insertions and deletions (indels). Half of the indels (11 of 22) occur in short mononucleotide tracts and paired tandem repeats, which may serve as hotspots for replication slippage (Clayton et al., 2016). Such mutations may occur independently in different lineages, distorting the phylogenetic signal, and in the analysis of genetic distances, we used only the information about nucleotide substitutions.

Three specimens had allele pair probabilities < 0.95 (Table 1), unique alleles from these specimens shown on the network with single alleles of *Parabaikalia dubiosa* and *Korotnewia semenkewitschii* (Fig.). Genotypes are sufficient for interspecific genetic distances estimates therefore rare alleles were not excluded from the analysis.

Genetic distances between 43 unique intron alleles in pairwise comparisons are from 0.19% to 3.86%. Intraspecific distances vary from 0 to 0.8% of nucleotide substitutions. Moreover, we found two shared alleles. The first one occurs in representatives of closely related *Korotnewia angygira* and *K. korotnewi* from different localities. The second shared allele has a central position in the phylogenetic network and was found in the members of the species *Teratobaikalia ciliata* and *Pseudobaikalia zachwatkini* which significant differ morphologically. The presence of the common alleles is likely owing to the retention of ancestral polymorphism.

Table. Ecological and teleoconch sculpture characters of Baicaliidae species and specimens examined in this study.

Species	Minimum depth	Maximum depth	Basin	Substrate	Hair	Ribs	Collection sites	GenBank no.
Baicalia carinata	15	100	a	m	n	n	Arul, Yakshakan, Semisosennaya	KF201695-KF201697
Baicalia dybowskiana	3	80	a	m	n	y	Semisosennaya	KF201698
Baicalia turriformis	10	40	s	r	n	y	Polovinnaya	KT885112
Godlewskia wrzesniowski	15	120	s	s	n	y	Olkhon gate	KF201707
Korotnewia angigyra	11	100	n	s	y	n	Barguzinsky gulf	KF201706
Korotnewia korotnewii	10	120	a	s	y	n	Olkhon gate, Senogda bay	KT885113, MT261207- MT261210
Korotnewia semenkewitschi	2	100	a	s	y	n	Barguzinsky gulf, Ayaya, Goloustnoe	KF201705, MT261211- MT261212*
Liobaikalia stiedae	15	120	s	s	y	n	Listvyanka, Utulik	KT885093, MT261218
Maackia herderiana	1	20	s	r	n	y	Listvyanka	KF201699
Maackia variesculpta	1	20	n	r	n	y	Arul	MT261226
Parabaikalia elata	4	20	a	s	n	n	Peschannaya bay	KF201704
Parabaikalia dubiosa	4	20	n	s	n	n	Boro-Elga, Olkhon gate, Nemnyanka	KT885102- KT885103*, MT261225*
Parabaikalia kobeltiana	5	30	s	s	y	n	Boro-Elga	KF201701
Parabaikalia oviformis	3	20	a	s	n	n	Peschannaya bay	KF201702, KF201703
Pseudobaicalia contabulata	10	40	a	s	n	y	Olkhon gate, Ayaya, Onokachanskaya	KT885109, MT261216- MT261217
Pseudobaicalia elegantula	1	15	n	m	n	y	Turkinskaya bank	MT261223
Pseudobaikalia jentteriana	15	100	n	s	n	n	Olkhon gate	KT885104, KT885105
Pseudobaikalia michelae	11	40	na	m	n	n	Kultuk, Barguzinsky gulf	KT885096-KT885101
Pseudobaikalia pulla	15	120	a	s	n	y	Olkhon gate, Listvyanka, Onokachanskaya, Svyatoy nos	KF201700, MT261224, KT885106-KT885108
Pseudobaicalia zachwatkini	3	30	a	m	n	y	Listvyanka, Onokachanskaya, Nizhneangarsk	KT885095, MT261213- MT261215
Teratobaikalia duthiersii	15	50	s	m	n	y	Kultuk	KF201709
Teratobaikalia ciliata	2	15	s	r	n	y	Polovinnaya, Listvyanka, Kedrovyy cape, Izhimey cape	KT885094, MT261219-MT261222

Basin: s – south; n –north; a – all area. Substrate: s – soft (mud and sand); r – hard; m – mixed. Hairs and ribs: y – present; n – not present. GenBank numbers of genotypes with allele pair probabilities <0.95 marked with an asterisk.

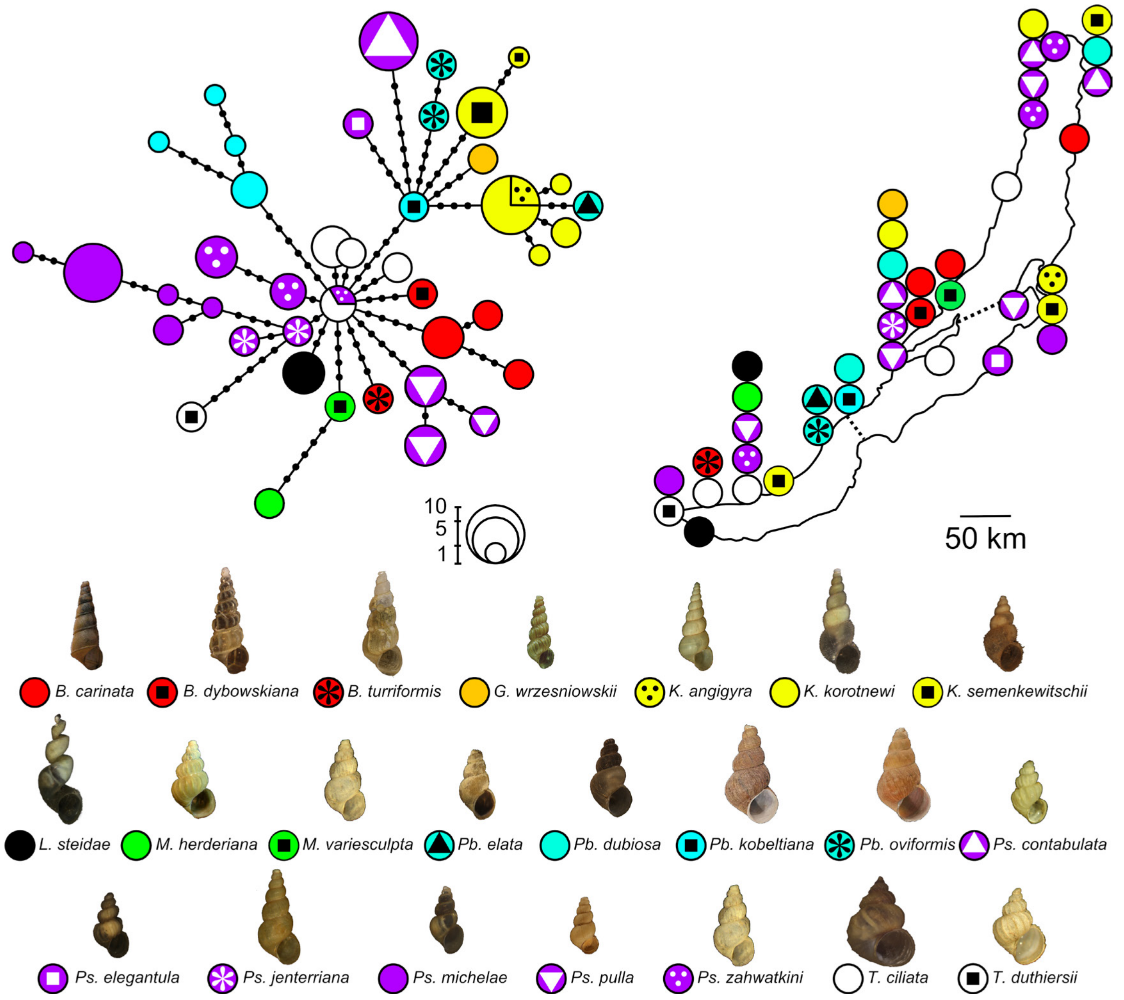

Fig.1. Intron allele network and localities for Baicaliidae species. Circle sizes are proportional to allele frequencies and colors represent the respective genera, species indicated by symbols. Dotted lines marked the boundaries of the Baikal basins.

Interspecific differences of intron sequences are small; at the same time, intraspecific polymorphism may be significant (Fig.). The star-like network topology allows to distinguish two genetic groups of baicaliids differing by at least four nucleotide substitutions.

Median interspecific distances are 1.36 ± 0.6% for the first group and 1.03 ± 0.65% – for the second group. The larger and more diverse genetically group includes 13 species: all members of the genera *Baicalia*, *Liobaikalia*, *Maackia*, *Teratobaikalia*, and *Pseudobaikalia*, except for *P. contabulata* and *P. elegantula*. In addition to the indicated exceptions, the second group includes the species of the genera *Godlewskia*, *Korotnewia* and *Parabaikalia*, except for *P. dubiosa*. Therefore, the members of two genera, *Parabaikalia* and *Pseudobaikalia*, appear in both genetic groups. Notably, only one and two species represent the genera *Godlewskia*, *Maackia* and *Teratobaikalia*, which is less than a third of their species diversity.

The species from the first group differ in their substrate preferences. Four of them are strict psammobionts: *L. stiedae*, *P. dubiosa*, *P. jentteriana*, and *P. pulla*; the other four belong to strict lithobionts: *B. turriformis, M. herderiana, M. variesculpta*, and *T. ciliata*. The remaining five species are found on various types of substrates (*B. carinata*, *P. elegantula* and *P. michelae*) or migrate during the breeding season from sand to stones (*B. dybowskiana* and *T. duthiersii*).

In the second group, only one of the nine species inhabits mixed-type substrates. *P. elegantula* inhabits the bottom side of large stones and boulders lying on the sand. Thus, this is a group with predominantly psammobiontic species. ML tree contains the same well-supported clades (BS = 77 for both).

The only factor that differentiates the interspecific distances is the preferred substrate of the baicaliids species (R2 = 0.35, $p = 0.018 < 0.05$ after correction). Minimum and maximum depths, shell sculpture or geographical distribution do not significantly discriminate phylogenetic species relatedness.

4. Discussion and conclusions

Intron allele network topology indicates two genetic groups of baicaliids. One group consists of the species adapted to highly diverse habitats on different sediments, while most members of the second group occupy various habitats on sandy substrates. Psammobiont species of both groups include species as able to burrow in soft substrates as species actively move along the surface of soft sediments. Ribs and periostracal hairs (or plates of various shapes) on the shell also occur in representatives of both groups. Additionally, the degree of manifestation of these morphological characteristics can vary at the intraspecific level (Kozhov, 1936; Sitnikova and Maximova, 2015). Therefore, adaptations to the same environments in the two groups could evolve independently. Data on the food composition of sandy living baicaliids is scarce (Roepstorf et al., 2003), meanwhile, by analogy with "rocky" species (Sitnikova et al., 2012), we suggest that co-existent "sandy" species consume different foods.

The species diversity of the psammobiont clade suggests the significance of the variety of microhabitats and the corresponding trophic adaptations that exist on soft substrates. One of the likely reasons for the formation of the observed diversity may be also preadaptation in the ancestor of psammobiont group, due to which it was more difficult for its members to adapt to habitat on the stony substrates.

One of the most interesting examples illustrating the influence of substrates on the diversification of species flock is cichlids from Lake Malawi where one of the genetic lineages inhabits sandy substrates, and another – the stony ones. A study of this species flock is explained by a three-stage hypothesis of the species flock development, according to which the speciation at the initial stage occurs when different substrates are occupied (Streelman and Danley, 2003). This is followed by separation according to trophic preferences, and at the final stage of the species flock diversification sexual selection takes place (Santos-Santos et al., 2015; Malinsky and Salzburger, 2016). The applicability of this model remains controversial (Hulsey et al., 2017), although it has become widespread for many groups of organisms (Ackerly et al., 2006; Leaché et al., 2016; Veríssimo et al., 2017). Perhaps, this example represents an individual case of phylogenetic niche conservatism (PNC) when closely related species occupy similar niches (Hopkins et al., 2014; Morinière et al., 2016).

The reasonable explanation for the evolution of the observed baicaliid diversity may be the change in the availability of different substrates during the development of the Baikal Rift. Estimates of the baicaliids divergence times about 2.5-4.3 (Zubakov, 1999; Mats et al., 2011) coincide with the beginning of the climatic and geomorphological changes (Kachukov et al., 1998; Mats and Yefimova, 2011). The terrestrial and underwater relief experienced great transformations ca. 4-3 which led to the sedimentation pattern changes as well as to a decrease in the availability of soft substrates, especially along the west coast (Mats and Yefimova, 2011). Over time, soft substrates restored, which could promote the diversification of a psammobiont clade.

In some other ancient lakes, the rapid speciation of endemic caenogastropods also coincided with great geological changes. For instance, significant changes in the level and salinity of Lake Titicaca and the preceding paleolakes coincide in time with the diversification of the species flock within the genus *Heleobia* (Kroll et al., 2012). In Lake Biwa, endemic gastropods of the genus *Semisulcospira* (*Biwamelania*) formed two species flocks whose ancestors appeared in the lake at different times. Nevertheless, the difference in niches (different types of substrates and depths) was simultaneous in both lineages in response to the lake expansion (Miura et al., 2019).

The composition of the two identified genetic groups of baicaliids does not fully correspond to the existing taxonomy and phylogenetic reconstructions based on a mitochondrial marker (Zubakov, 1999; Sitnikova, 2004). Though both molecular phylogenies show the relatedness of members of the genera *Korotnewia* and *Parabaikalia* as well as polyphyly of the genus *Pseudobaikalia*. Previous phylogenetic analysis of the intron of ATP- synthase alpha-subunit gene from 11 baicaliid species shows similar results (Kovalenkova et al., 2015). However, extended data set presented here shows that even very morphologically different species can have small genetic distances maybe due to the retention of ancestral polymorphism (for example in the case of *P. zahwatkini* and *T. ciliata*).

However, our phylogenetic conclusions are also based on one genetic locus, and the existing evolutionary relationships between sister species may not be correctly interpreted. There are several examples of mismatch in the degree of genetic relatedness of baicaliids evaluated using markers with different cellular localization (Peretolchina et al., 2007; Sitnikova et al., 2016; Sherbakov et al., 2017). Obtaining the most complete information about the processes of baicaliid speciation requires the use of several variable and unlinked genetic markers.

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