New Approaches to Study Biodiversity and Evolution of Freshwater Sponges in Ancient Lakes.

Valeria Itskovich1*, Sergey Belikov1, Yoshiki Masuda1, Tsutomu Nohno1, Sofia Efremova2, Martin Meixner4 and Dorte Janussen5.

1Limnological Institute of the Siberian Branch of The Russian Academy of Sciences, Ulan-Batorskaya 3, 664 033 Irkutsk, Russia
2Laboratory of Ontogenesis, Biological Research Institute, St. Petersburg State University, Oranienbaumskoe sch. 2, Stary Peterhof, 198 904 St. Petersburg, Russia;
3Department of Biology, Kawasaki Medical School, Kurashiki, 701-0192, Japan;
4Institute fur Genetic, Humbold-Universitat zu Berlin, Germany;
5Forschungsinstitut und Naturmuseum Senckenberg, Frankfurt am Main, Germany.
*Corresponding author; Email: itskovich@mail.ru

ABSTRACT

Freshwater sponges (Porifera; Spongillina) include 6 families. Some of them are endemic of ancient lakes Baikal, Tanganyika, Ohrid and Malawi. Others families are cosmopolitan and inhabit inland waters worldwide. Freshwater sponges are important for cleaning of water due their filter activity and are often main part of benthos in the lakes. The taxonomy of freshwater sponges is difficult that complicates an estimation of their biodiversity. Evolution and phylogenetic relationships of these animals also are not well known.

Freshwater sponges from lakes Baikal (family Lubomirskiidae), Biwa (family Spongillidae) and Tanganyika (family Potamolepidae) were collected during several Japan-Russian joint expeditions and analyzed by morphology and molecular methods. Several gene regions (18SrRNA gene, COXI gene and internal transcribed spacers of rRNA) were sequenced and analyzed. The obtained results allowed to develop a molecular marker for species identification in Porifera and to study the phylogenetic relationships between endemic and cosmopolitan freshwater sponges.

Our results indicated that the endemic family Lubomirskiidae is monophyletic, showing the highest bootstrap support. The genetic distances between Lubomirskiidae species are much lower than those between Spongillidae species. The lower genetic distances between species of Baikalian sponges is indicative of their relatively recent radiation from a common ancestor. The evolutionary age of the family Lubomirskiidae does not exceed the geological age of Lake Baikal, which indicates an autochthonous evolution of sponges in Lake Baikal. The cosmopolitan family Spongillidae is paraphyletic in relation to other freshwater sponges. The genera Radiospongilla and Eunapius are probably monophyletic and the genus Ephydatia is paraphyletic and forms basal branches. The family Potamolepidae is more closely related to common ancestor of all freshwater sponges.

The developed molecular method can be useful for identification of species of freshwater sponges. Study of species diversity of this important group of organisms will allow performing an ecological monitoring of lake ecosystems.

Keywords: Spongillina, Phylogeny, ITS, Evolution, Molecular taxonomy, Baikal, Biwa, Tanganyika

INTRODUCTION

Sponges (Porifera) are ancient metazoans which mainly inhabit marine ecosystems but also have colonized freshwater environments (Willmer 1990; Nielsen 2001). Their phylogeny and taxonomy are complicated due to a limited amount and variability of morphological signatures useful for taxonomy (Hooper and van Soest 2002). The freshwater sponges consist of six extant and one fossil family belonging to the suborder Spongillina, which are classified together with marine families having diactinal megascleres to comprise the order Haplosclerida Topsent (Mancony and Pronzato 2002). Several families are endemics of ancient lakes Baikal, Tanganyika, Ohrid and Malawi. The family Spongillidae is cosmopolitan and includes 21 genera with more than 150 species (Penney and Racek 1967; Mancony and Pronzato 2002). The family Lubomirskiidae inhabits Lake Baikal (Siberia), the deepest and oldest lake in the world, which houses a great number of endemic species and is a place of intense evolutionary processes (Timoshkin 1995). Recently the number of described species of Lubomirskiidae has appreciably increased and at present 14 species and 1 subspecies of this family are known (Efremova 2001, 2004).

Freshwater sponges are important for cleaning of water due their filter activity and accumulate not only nutrients but also pollutants. The high degree of bioaccumulation of pollutants is one of the reasons...
why sponges are considered as suitable bioindicators to detect environmental pollution.

The taxonomy of sponges is based on the structure of their skeleton and its structural elements, megascleres and microscleres. Some species of sponges produce asexual resting bodies (gemmules), which contain specialized microscleres (gemmae). For the classification of freshwater sponges, presence and form of microscleres, and the structure of gemmules and gemmules considered the most important diagnostic features. However, species of three of the six extant freshwater sponge families, Lubomirskiidae, Malawispongiiidae, Metchnikowidae, do not have such important morphological diagnostic signatures as microscleres, gemmules and, accordingly, gemmules (Mancony and Pronzato 2002). Therefore, in the case of these species, classification can be based only on the forms of megascleres and the characteristic features of skeletal organization, but these features are highly variable. Since gemmules are facilities for survival under adverse conditions of habitat, sponges produce gemmules during a restricted time of the year (Mancony and Pronzato 2002). As a result, even in Spongillidae species that are able to produce gemmules, these are often absent in collected samples, making their species identification impossible. These factors determine the urgency of using molecular biological methods to study the phylogeny of freshwater sponges and developing convenient molecular markers for their species identification.

Many authors considered freshwater sponges to be polyphyletic and believed that the sponges of ancient lakes and cosmopolitan freshwater sponges would be derived from different ancestors. This idea has been supported by Marshall (1885), Brien (1970), Volkmer-Ribeiro and De Rosa-Barbosa (1978), Volkmer-Ribeiro and Watanabe (1983) and Volkmer-Ribeiro (1990). However, the first molecular approaches revealed a possible monophyly of freshwater sponges, although only several species from half of the existing families has been examined (Itskovich et al. 1999; Efremova et al. 2002; Schröder et al. 2003; Addis and Peterson 2005; Itskovich et al. 2006). Since the first molecular biology studies were based mainly on the conservative 18S ribosomal RNA gene and on the in Porifera slowly evolving cytochrome oxidase subunit I gene, they have been useful only for the families and at upper levels. Therefore, the phylogeny within freshwater sponge families remains unclear.

The ITS region is convenient for phylogenetic studies because of the high variability of spacers and the possibility of verifying and aligning sequences according to the conservative parts of ribosomal RNAs. Verification of the obtained sequences is extremely important for sponges, because they are inhabited by a great number of symbionts, including intracellular symbionts (Wilkinson 1978). The ITS region is one of the most variable parts of the genome and is suitable for the analyses of closely related species (Coleman et al. 2002; Coleman 2003). ITS spacers have been successfully used for phylogenetic reconstructions in plants, insects and animals including other diploblast, Cnidaria (Yoon et al. 2001; Bargues et al. 2001, 2003; Young et al. 2004).

Studies of the ITS region in sponges that have recently appeared have been concerned with analyses of the phylogenetic histories of marine sponge populations (Warheide et al. 2002; Duran et al. 2004) and phylogenetic study of the marine family Aplysinidae (Schmitt et al. 2005). In a study by Addis and Peterson (2005), analyses of the ITS2 sequences of two species of Lubomirskiidae and two species of Spongillidae revealed possible paraphyly of Spongillidae in relation to Lubomirskiidae. However, due to the restricted number of species analyzed, this conclusion was preliminary.

In this work we carried out analyses of the ITS1 and ITS2 regions of 11 species of Lubomirskiidae, 13 species of Spongillidae and 1 species of Potamolepidae to investigate the phylogenetic relationships between and within these families and the development of molecular markers for species identification in sponges.

MATERIAL AND METHODS

Sample collection and DNA extraction

Species of freshwater sponges belonging to the family Spongillidae were collected in Lake Biwa and other lakes, rivers and canals of Honshu island (Japan). Samples were collected manually from shallow depths. Samples of the family Lubomirskiidae were collected during Japan-Russian joint expeditions to Lake Baikal in 1993-2004. The collection sites are situated in the litoral zones of south, medium and north Baikal. Sample collection was carried out by scuba diving and by dredge surveys from depths, of 1 to 200 meters. More than 1300 samples of the family Lubomirskiidae were collected. Specimens of Echinospogilla brichardi (Potamolepidae) were collected from Lake Tanganyika (Zambia).

For molecular analyses, samples of 11 species of Lubomirskiidae, 13 species of Spongillidae and 1 species of Potamolepidae were used. The taxonomy and collection sites are shown in table 1.

All specimens were photographed alive. Data on ecology, habitat and texture were recorded. One part of each sample was fixed in 70% ethanol for taxonomic identification, and another part was frozen in liquid nitrogen for molecular analysis or was used for DNA extraction immediately.
Table 1. List of species examined with collection sites, lengths of ITS spacers and accession numbers of obtained sequences.

<table>
<thead>
<tr>
<th>Species</th>
<th>Voucher</th>
<th>Collection</th>
<th>Length of ITS1</th>
<th>Length of ITS2</th>
<th>Accession N°</th>
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<td>Verkhnee izgolovje, Baikal, 10m</td>
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<td>Bolshtie Ushkany, Baikal, 23m</td>
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<td><em>Echinospongilla brichardi</em></td>
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<td>Lake Tanganyika</td>
<td>210</td>
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<td>EF418032</td>
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</table>

Species identification was performed based on microscopic study of the skeleton. Spicule and skeleton preparation were performed as previously described (Masuda et al, 1999; Efremova 2001).

Total genomic DNA extraction was performed from internal, symbiont-free parts of the sponges by the standard phenol method (Sambrook et al. 1989), the CTAB method (Gustincich et al. 1991) and by using the Genomic–tip 100 G Kit (QIAGEN). DNA was analyzed by electrophoresis in 0.6% agarose, and its concentration was measured by a spectrophotometer. Purification of DNA was carried out using Nucleosil sorbent (MACHEREY–NAGEL).

**Polymerase chain reaction, cloning and sequencing**

The primer design was made using the program GeneTools (Resenchuk, 1991). The following primers were chosen based on the conservative
regions 18S pPHK and 28S pPHK of marine sponge species from GenBank:
Forward primer (5w13)
5'- TACACACCCTCTGCTGCTGTTTCTACGACGGATAGTGGGGAGGTTTGGCTTAAGAGGTTTT
Revers primer (1278)
5'- CTYYGACGTGCCTTTCCAGGTTG

Fragment of 1060 bp length including a 3' end of 18S rRNA, ITS1, 5.8S rRNA and a 5' end of 28S rRNA, were amplified. 25μl PCR reaction mix contained 10μlPCR Buffer (Promega) –2.5μl MgCl2 (25mM) -3μl, each primer (10pmol/μl) - 0.5μl, dNTP mix (100mM each) -1μl, DNA (~0,1μg) -1μl, and Taq DNA polymerase (5u/μl) (Promega) - 0.2μl, ddH2O - to 25μl. The cycle parameters were: initial denaturation at 94oC for 120secs, followed by 40 cycles of denaturation at 94oC for 60secs, anneling at 55oC for 60secs, and extension at 72oC for 60secs, followed by a final extension of 8mins at 72oC. Amplifications were carried out in a Perkin-Elmer amplificator. Each PCR reaction was purified through a QIAquick Spin column (GIBA) and cloned into pGEM-T (Promega). Sequencing of the clones was carried out by a ABI 373A automatic DNA Sequencer (Applied Biosystems) according to protocols. Chromotogram fragments of about 1060 bp in length including 77 bp of a 3' end of 18S rRNA, 153 bp of 5.8S rRNA and 114 bp of a 5' end of 28S rRNA. The ITS1 and ITS2 lengths varied between analyzed freshwater sponge species, varying from 215 to 260 bp for ITS1, and from 237 to 294 bp for ITS2 (table 1).

**Phylogenetic analyses.**

The structures obtained were analyzed with the BLAST program (Alischul et al. 1997) and aligned with the CLUSTAL W program (Thompson et al. 1997). Fifty-six different models of evolution were screened by using the MODELTEST program (Posada and Crandall 1998). Maximum-likelihood (ML), neighbor-joining and maximum-parsimony analyses were done using the programs MEGA 1.01 (Kumar et al. 1993), TREEPUZLE (Schmidt et al. 2000) and MrBayes v3.0 (Ronquist et al. 2003). The distance estimation was carried out using the formula of Kimura (1980). Bootstrap values were calculated from 500 replicates. The sequence of the marine species order Haplosclerida *Haliclona amphioxa* (GenBank accession no. AJ703887) was used as the outgroup. The distance estimation and phylogenetic trees were obtained for two sets of data: one set included all the obtained sequences of Spongillidae and the other second set included only consensus sequences for each analyzed species.

**RESULTS**

Sequences of the ITS region of 11 species of Lubomirskiidae, 13 species of Spongillidae and 1 species of Potamolepidae were obtained. For some species of Spongillidae, several samples from different localities were analyzed, and the common amount of analyzed samples was 39. Fragments were from 797 bp (*Echinogorgilla brichardi*) to 953 bp (*Baikalospongia intermedia*) in lengths including 77 bp of a 3’ end of 18S rRNA, 153 bp of 5.8S rRNA and 114 bp of a 5’ end of 28S rRNA. The ITS1 and ITS2 lengths varied between analyzed freshwater sponge species, varying from 215 to 260 bp for ITS1, and from 237 to 294 bp for ITS2 (table 1).

**Figure 1.** Fragment of alignment of the obtained sequences showing Lubomirskiidae specific insertions.
The ITS sequences of the Lubomirskiidae species were larger in size than those of the Spongillidae species and Potamolepidae species. The larger size of ITS1 and ITS2 in Lubomirskiidae resulted from several insertions up to 26 bp located in the ITS1 and in ITS2 regions (Fig. 1). The largest differences were situated in the ITS1 and ITS2 regions of rRNA, although whereas the regions of 18S and 28S rRNA were conservative. The average GC content in the obtained sequences was 55% and the total nucleotide composition was A-22.3%, C-26.1%, G-28.9%, T-22.7%. The nucleotide composition and GC content of the obtained sequences of freshwater sponges were similar to those in marine sponge species (Wörheide et al. 2002; Duran et al. 2004).

The genetic distances in pairwise comparisons between all analyzed samples were calculated according to Kimura’s two-parameter model. The average homology between the sequences obtained from Spongillidae species was 69%. Between Lubomirskiidae species sequences the mean level of homology was extremely high (96.4%) in comparison with Spongillidae.

Since, for some Spongillidae species ITS sequences were obtained from several samples from different localities, we could estimate the level of intraspecies variability of this DNA region within one species. The genetic distances between different samples of one species were calculated. The genetic distance between different samples within H. multidentata was 0.1 %, E. fluviatilis - 1.5 %, R. cerebellata - 0.2 %, S. lacustris - 0.6%. The obtained data revealed that the homology between sequences of the ITS region within one species of Spongillidae varied from 98.5 to 99.9%.

Two sets of data were analyzed. One set included the consensus sequences for each analyzed species, and the marine species from the order Haplosclerida Haliclona amphioxia (GenBank accession no. AJ703887) was used as the outgroup. The length of alignment was 1076 bp, 647 nucleotide positions were variable, and 379 nucleotide positions were parsimony informative. For better resolution of the relationships within Spongillidae, all the obtained ITS sequences of Spongillidae samples were included in the second set of data and rooted by the sequence of Trochospongilla latouchiana. In the 1011 bp long alignment 398 sites were variable and 304 were parsimony informative. The results of using the MODELTEST program (Posada and Crandall 1998) revealed that general time-reversible (GTR) model incorporating estimates of the proportion of invariable sites (I) and gamma is best model for our data.

Dendrograms obtained by neighbor-joining, maximum-parsimony and maximum-likelihood methods had similar topology. There were small differences in the clustering of closely related species within the family Lubomirskiidae, but the bootstrap support of these relationships was very low and can not be discussed. Therefore only the neighbor-joining tree is shown for both datasets (Fig.2, 3). The family Lubomirskiidae is strongly monophyletic on all phylogenetic trees. The family Spongillidae is monophyletic (55%-NJ, 74%-MP, 79%-ML) with the exception of Trochospongilla latouchiana. Divergence of freshwater sponge families from a common ancestor according to the topology of the phylogenetic trees obtained took place in the following order: Potamolepidae with Trochospongilla latouchiana, Spongillidae, Lubomirskiidae (Fig. 2). Clusterization of the Spongillidae species was similar on the trees obtained by different methods. There are several well supported monophyletic groups among the species of this family. The group including the genera Eunapius and Spongilla is supported on the tree obtained for the first dataset (85%-NJ, 92%-MP, 77%-ML) and for the second dataset (95%-NJ, 83%-MP, 100%-ML). Spongilla lacustris branched from the base of a group including a species of Eunapius genera that revealed the existence of a common ancestor of Eunapius and Spongilla. The phylogenetic relationships of Radiospongilla with other freshwater sponge genera are unresolved.

The monophyletic group of Eunapius consists of Eunapius coniferus, Eunapius ryuensis, Eunapius sinensis, Eunapius sp1 and Eunapius sp2. Three species comprise the Radiospongilla group: R. sendai, R. cerebellata, R. crateriformis. According to these data, the genera Eunapius and Radiospongilla are monophyletic.

Two species of Ephydatia – Ephydatia fluviatilis and Ephydatia muelleri, and also Heterorotula multidentata are situated at the base of the phylogenetic tree, and their relationships with other species of Spongillidae have not been resolved (Fig. 2) or they form a separate monophyletic group with high bootstrap support (99%-NJ, 97%- MP, 100%-ML) (Fig.3).

On the obtained phylogenetic trees, all species of the family Lubomirskiidae formed a monophyletic clade with highest bootstrap support (100%-NJ, 100%- MP, 99%-ML). Within this monophyletic group, the phylogenetic relationships are unresolved because of the low level of divergence between the sequences of the ITS region. Almost all branches within the clade of Baikalian endemic species had low levels of bootstrap support. The distribution of the species within the four existing genera was not supported by tree topology. The species of genus Baikalospongia were distributed on different groups and also the species of Lubomirskia did not form a monophyletic group, but these relationships have low support. It is interesting finding that an undescribed species with spicules which morphologically similar to Spongillidae (voucher BK267) clustered with the Lubomirskiidae clade (81%-NJ, 78%- MP, 71%-ML) (Fig. 2).
All other species of the family Lubomirskiidae are characterized by an extremely high level of genetic similarity and unresolved phylogenetic relationships between them.

Figure 2. Neighbour-joining phylogenetic tree based on the obtained ITS sequences of all analyzed species. Maximum parsimony and maximum likelihood trees have similar topology. The numbers under the nodes are bootstrap values for NJ, MP, ML analyses (from top to bottom). The tree is rooted on the marine haplosclerid species *Haliclona amphioxa* (GenBank accession no. AJ703887).

Lubomirskiidae  Spongillidae  Potamolepidae
DISCUSSION

Phylogeny of Spongillidae

Spongillidae are the biggest freshwater sponge family, including more than half of all the existing species of the suborder Spongillina (Mancony and Pronzato 2002). The taxonomy of Spongillidae is based on the forms of microscleres and gemmoscleres. Megascleres are mainly similar in form and size in different species but vary within one species (Penney and Racek 1967; Mancony and Pronzato 2002). Racek and Harrison (1975), based on paleontological data, supposed that the family Spongillidae is monophyletic. In their opinion, the genus *Radiospongilla* is ancestral for the remaining genera of Spongillidae. However, these conclusions are not supported by our results based on ITS sequences.

All together, six of 21 existing genera of the Spongillidae were analyzed. Our analysis of the ITS region revealed that all the studied species of the family Spongillidae clustered in one monophyletic group with the exception of a species from genus *Trochospongilla: T. latouchiana*. branches together with *Echinospingilla brichardi* from the family Potamolepidae, but the bootstrap support of this clade is not high (NJ- 52%, MP- 60%, ML -62%).

Figure 3. Neighbour-joining phylogenetic tree based on the obtained ITS sequences of all analyzed Spongillidae samples. Voucher numbers are indicated in in brackets. Maximum parsimony and maximum likelihood trees have similar topology. The numbers under the nodes are bootstrap values for NJ, MP, ML analyses (from top to bottom). The tree is rooted on the *Trochospongilla latouchiana* (Spongillidae).

* Sequences obtained from the GenBank (Ephydatia fluviatilis- GenBank accession no. AJ705048; Spongilla lacustris - GenBank accession no. AJ703890).
Therefore, according to our data, the family Spongillidae is not monophyletic. These results suggest that the phylogeny of the suborder Spongillina on the family level should be reexamined after additional molecular data from a larger number of species have become available.

Branching of the phylogenetic trees within a group combining all species of the family Spongillidae is in general in accordance with the morphology-based taxonomy of this family. The clade including species of the genus *Eunapius*: *E. sinensis*, *E. ryensis* and *E. coniferus* had the highest bootstrap support – 98%-100%. Species of the genus *Radiospongilla*: *R. sendai*, *R. cerebellata*, *R. crateriformis* also comprise one monophyletic group with bootstrap support of 58%-96% in different data sets (Fig. 2, 3). The obtained data provide evidence for the monophyly of the genera *Eunapius* and *Radiospongilla* which is in accordance with morphological taxonomy data (Penney and Racek 1968; Pronzato and Mancony 2001). For *Spongilla*, only one species was analyzed, so we could not test the monophyly of this genus.

Among the studied freshwater sponges, the genus *Ephydatia* is situated most basal, showing its close relationships with the common ancestor of Lubomirskiidae and Spongillidae (Fig.2). The monophyly of the genus *Ephydatia* was not supported – *Ephydatia fluviatilis* and *Ephydatia muelleri* do not cluster together, but *E. muelleri* is situated together with *H. multidentata*. Their relationships with other Spongillidae were not resolved (Fig.2). On a tree based on all the sequenced samples, a clade including *Ephydatia fluviatilis*, *Ephydatia muelleri* and *Heterorotula multidentata* had high bootstrap support (97-100%) (Fig.3). This branching supports the possible paraphyly of *Ephydatia* in relation to *Heterorotula*. The synapomorphic characters for this clade are absence of microscleres, radially arranged birotulate gemmuloscleres and amphioxea megascleres (Fig. 4).

*Spongilla lacustris* branches from the base of a group including species of the genus *Eunapius* which revealed close relationships with the common ancestor of Lubomirskiidae and Spongillidae (Fig.2). The monophyly of the genus *Ephydatia* was not supported – *Ephydatia fluviatilis* and *Ephydatia muelleri* do not cluster together, but *E. muelleri* is situated together with *H. multidentata*. Their relationships with other Spongillidae were not resolved (Fig.2). On a tree based on all the sequenced samples, a clade including *Ephydatia fluviatilis*, *Ephydatia muelleri* and *Heterorotula multidentata* had high bootstrap support (97-100%) (Fig.3). This branching supports the possible paraphyly of *Ephydatia* in relation to *Heterorotula*. The synapomorphic characters for this clade are absence of microscleres, radially arranged birotulate gemmuloscleres and amphioxea megascleres (Fig. 4).

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The presence of monophyletic groups corresponding with morphological taxonomy supports the usefulness of the ITS region for phylogenetic studies of sponges. We also analyzed samples from Lake Biwa and Shintone river for which identification based on morphological features was difficult (vouchers BW0107, JP18, BW0115). After analyses of the ITS region, these samples were identified as *Heterorotula multidentata*.

Systematic study of the genus *Eunapius* remains difficult (Masuda and Satoh 1992). Five samples of *Eunapius*, identification of which by morphological methods was impossible, were analyzed. Four of them (vouchers BW0114, BW0101, JP9, BW0104) marked as *Eunapius* sp. 2 (Fig. 3) had almost identical sequences of ITS1 and ITS2 (99% of homology), whereas the sequence of the fifth sample (voucher BW0118), marked as *Eunapius* sp.1 (Fig. 3), differed significantly. These samples had sequences that differed from other analyzed species of the genus *Eunapius*. *Eunapius* sp. 2 had homology of 97% with *Eunapius sinensis*, and *Eunapius* sp.1 – homology of 98% with *Eunapius coniferus* and *Eunapius ryensis*. Differences in the forms of spicules (Masuda, unpubl.) and the obtained molecular data suggest that these sponges should be described as a new species or subspecies belonging to genus *Eunapius*.

There is a very serious problem of species identification for freshwater sponges, because the absence of gemmules often makes it impossible to determine their exact taxonomic position. Our obtained sequences of ITS1 and ITS2 showed the existence of substitution between the sequences of all different species in this genomic region. Intraspecific differences were never more than 1.5 % and never exceeded interspecific variability (Fig.3). This
indicates the possibility of effectively using this region for the species identification of freshwater sponges and consequently for estimation of their biodiversity. A sufficient amount of substitution would permit the use of short genome fragments (200–300 bp) for species identification, which would be easier and cheaper than sequencing of the 18S ribosomal RNA gene (Sipkema et al. 2003).

According to our results from several Spongillidae species, most of the ITS1 and ITS2 sequences of samples from different localities belonging to one species have nucleotide substitutions. The ITS sequences have been successfully used for studies of the population history of marine sponges (Duran et al. 2004). It was considered that in spite of multicopy of ITS identity of copies is remained due to concerted evolution, however, in marine sponges intragenomic variability of ITS was revealed that can interfere with population level studies (Worheide et al. 2004). Probably the ITS region can also be used for population studies of freshwater sponges, but as in the case of marine species, screening for intragenomic polymorphism should be done for each populations before study.

Phylogeny of Lubomirskiidae

The sponges of the family Lubomirskiidae are endemics to Lake Baikal with the exception of a species from Lake Chagytai (Rezvoj 1936), whose affinity to this family should be checked by molecular data in the future. This family was created by Rezvoj (1936) on a base of their anatomy, skeletal peculiarities and the absence of the gemmules. He considered Lubomirskiidae to be monophyletic and to probably be descendants of marine families. Therefore, he concluded that the origin of Lubomirskiidae bears no relation to Spongillidae. In the first work on the molecular phylogeny of freshwater sponges based on partial sequences of the 18S rRNA gene, a possible non monophyletic status for Lubomirskiidae was suggested, but this conclusion was based on single substitutions in sequences and had low bootstrap support (Itskovich et al. 1999). Single species of Spongillidae and Lubomirskiidae were taken for molecular analyses based on three different genes (Addis and Peterson 2005) and possible paraphyly of Ephydatia in relation to Lubomirskiidae was found. Therefore, up to the present study, it has been unclear whether Lubomirskiidae is monophyletic. In this work, we analyzed for the first time 11 species from 14 existing species which belong to all four genera of Lubomirskiidae. Based on our results of analyses of the ITS region, it was determined that the family Lubomirskiidae is monophyletic with the highest bootstrap support on phylogenetic trees obtained by different methods. These results clearly indicate that all present Lubomirskiidae species are derived from one ancestor which moved into Lake Baikal in the past.

Some assumptions about the phylogenetic relationships within Lubomirskiidae were made by Rezvoj (1936) based on different skeletal structures. In his opinion, the genus Baikalospongia is most primitive, because it has an irregular skeleton without main and secondary bunches. He considered that the genera Swartschewskia having a regular net of bunches in superficial layer and the genus Lubomirskia, with a firm highly differentiated skeleton are more evolutionarily advanced. On our phylogenetic trees all Lubomirskiidae formed strictly supported separate groups with considerably lower interspecies genetic distances between species than the distances between Spongillidae species. Due to the lower genetic distances, phylogeny within Lubomirskiidae is unresolved and the bootstraps within this group mostly do not exceed 50%. Distribution of the four genera according to the present classification is not supported by molecular data. Recently it was shown by analyses of 18S rRNA (Itskovich et al. 1999) and COXI genes that among Lubomirskiidae S. papyracea is diverged from a common ancestor earlier than other species (Efremova et al. 2002; Schröder et al. 2003;
Itskovich et al. 2006). Therefore, these data do not support the opinion of Rezvoj (1936) regarding the primitive status of the genus Baikalospongia. According to our ITS region analyses, S. papyracea may be placed in one group with other species and it is not distinguished by a larger genetic distance. This result may be related to the inconstancy of the evolution rate of the ITSs in comparison with the coding parts of genome used in previous studies. Because of the low level of divergence, it was impossible to clarify the phylogeny within Lubomirskiidae. However, since there are nucleotide differences between the analyzed species, ITS sequences can be used for molecular identification for both Spongillidae and Lubomirskiidae species. The ITS1 and ITS2 sequences are more suitable for species identification within Lubomirskiidae than the sequences of the 18S rRNA and COXI genes because on these parts of the genome most species of Lubomirskiidae were found to have identical sequences (Itskovich et al. 1999; Efremova et al. 2002; Schröder et al. 2003).

Addis and Peterson have recommended that Lubomirskiidae should be abandoned and have suggested that all lubomirkiid species belong to the genus Ephydatia (Addis & Peterson 2005). The evidence from our tree indicates that Spongillidae are paraphyletic in relation to all Lubomirskiidae species. However, according to our data, Lubomirskiidae and Spongillidae have formed two monophyletic groups and the genetic distances between them exceed distances between most other genera within Spongillidae, except T. latouchiana. Therefore, although we consider that Lubomirskiidae is closely related to Spongillidae, it should remain in a family rank. Until more molecular data become available, we suppose that the composition of families within the suborder Spongillina will be retained.

Differences in the form of spicules and skeletal patterns are the reasons for giving specific status to the Lubomirskiidae species (Efremova 2001, 2004) (Fig. 5). Calculations based on the ITS1 and ITS2 sequences show that the genetic distances between Baikalian sponge species reveal a higher level than the level of interpopulation differences within other species. In marine species, the level of intraspecific variability of ITS1 and ITS2 within Crambe crambe is 0.46-1.7% (Duran 2004), and within Leucetta chagonensis - 0.1-1.6% (Worheide 2004). For species of Spongillidae, according to our data, the intraspecific variability of ITS1 and ITS2 is equal to 0.1-1.5%. The mean level of genetic divergence between Lubomirskiidae species is about 3.6%. Although among the analyzed species there were no species with identical ITS sequences, the differences between them sometimes were restricted to a single substitution and deletion. However, analyses of other gene regions (18S and COXI) revealed a clear separation of at least one species (S. papyracea) from others (Itskovich et al. 1999; Efremova et al. 2002; Schröder et al. 2003; Itskovich et al. 2006). Our data either supports the hypothesis of a recent species divergence within Lubomirskiidae, or questioned their specific status. The finding of undescribed species of Lubomirskiidae (voucher BK267) revealed that the systematic of this family is not completed.

The low variability between species of Lubomirskiidae shows that their divergence took place later than that between species of Spongillidae. The lower divergence may be related to slower evolution of ITS in Lubomirskiidae than in Spongillidae, or indicate that extant Lubomirskiidae are an evolutionarily young species-group. Such rapid evolution was noted for many other benthic organisms in Lake Baikal, e.g., worms and mollusks (Zubakov et al. 1997; Sherbakov et al. 1999; Kaigorodova et al. 2000). Some early authors considered Lubomirskiidae to be relics of ancient freshwater or marine fauna (Berg 1900; Vereshagin 1935; Rezvoj 1936). Martinson described the discovery of spicules of Lubomirskiidae in sediments of the Late Oligocene Age (Martinson 1936).

Analyses of ITS sequences clearly indicated that divergence within Lubomirskiidae took place relatively recently and the evolutionary age of these species apparently does not exceed the geological age of Lake Baikal, which is considered to be about 30 million years (Mats 1993). Thus data from the ITS sequences analyses support claims of an autochthonous radiation of sponge species in Lake Baikal (Makushok 1925; Efremova et al. 1989). Divergence of Lubomirskiidae species flock took place after Baikal had already become a deep-water basin and probably it is the result of adaptation to a variety of ecological niches which came into existence within the lake. Another reason for the low level of genetic divergence between Lubomirskiidae species may be a relatively recent bottleneck event in the evolutionary history of this family. This can explain the long distance between Lubomirskiidae and Spongillidae and short distances between extant Lubomirskiidae species. This assumption is supported also by the results of paleontological studies. According to the results of spicule analyses, in the Late Pliocene there was wider species diversity including fossil species, than exists in Lake Baikal at the present time (Weinberg 2001).

Our results revealed the ITS region to be useful for study of the taxonomy and phylogeny of freshwater sponges and we suppose that future studies of this gene region will help to clarify their phylogenetic history. Wider analyses including other species and families of the freshwater sponges and analyses of population structures are in progress.

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