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Intraspecific and interspecific sequence variability in the ITS region of the rDNA of freshwater sponges of Lake Baikal and East Siberia

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ABSTRACT

Sponge (Porifera) systematics and phylogeny are complicated due to the limited number of morphological features and lack of information employed for taxonomy. Baikal sponges occur in the families Lubomirskiidae and Spongillidae, with 13 and 4 species in each family, respectively. The aim of this research was to study the variability of internal transcribed spacers (ITS) of rDNA in the families Lubomirskiidae and Spongillidae to determine the effectiveness of using ITS sequences to differentiate species and study the evolution of sponges in Lake Baikal. ITS1 and ITS2 (~950 bp) were amplified and sequenced for 34 samples of Lubomirskiidae and 28 samples of Spongillidae. Phylogenetic reconstructions revealed monophyly of *Ephydatia fluviatilis*, *Ephydatia muellery*, and *Spongilla lacustris*. Intraspecific polymorphisms were much lower than interspecific variability among the species of Spongillidae, despite analyzed samples of each species originating from geographically distant locations. Our findings revealed that ITS1 and ITS2 sequences could be an appropriate tool for phylogenetic study and species identification of Spongillidae. ITS analyses did not support monophyly of the lubomirskiid genera and species, possibly because of incomplete species divergence, indicating that species delimitation is difficult in the case of rapid species radiation within Lubomirskiidae.

KEYWORDS

Baikal; evolution; freshwater sponges; ITS1 and ITS2; Porifera; species identification

Introduction

Sponges (Porifera) are the most primitive multicellular animals and dominate the benthic biomass of many waterbodies, including Lake Baikal. As sedentary biofiltrators, sponges play an important role in natural water purification and, because of bioaccumulation, can be used as bioindicators in an environmental assessment. Sponges are also a source of many biologically active substances, making it imperative to develop the correct taxonomy for this group (Newman and Cragg 2012).

Sponge systematics and phylogeny are complicated because of the limited number of morphological features employed for taxonomy (Hooper and van Soest 2002). Most sponge species inhabit marine ecosystems, but approximately 200 species belonging to 45 genera have colonized freshwater environments (Manconi and Pronzato 2002).

Many species of freshwater sponges have the ability to produce gemmules, which contain totipotent cells and are resistant to extreme fluctuations of environmental conditions. The structure of gemmules and their skeletal elements, gemmoscleres, are crucial for species identification of sponges. The shape and dimensions of the basic elements of the skeleton, megascleres, vary depending on environmental conditions, and additional spicules, microscleres, are absent in many species.

Baikal sponges are represented by 2 families: Spongillidae and Lubomirskiidae. Family Spongillidae Gray 1867 is the largest of the families of freshwater sponges. At present, more than 150 described species of this family span 21 genera (Manconi and Pronzato 2002). The family Spongillidae includes cosmopolitan species that extend from the Antarctic to the tropics, including ancient lakes such as Baikal in Russia, Tanganyika in Africa, and Biwa in Japan.

The endemic family Lubomirskiidae Rezvoi, first described in 1936, is dominant in Lake Baikal and is represented 13 species and 2 subspecies (Efremova 2001, 2004; Itskovich et al. 2015). The Lubomirskiidae family with its associated species represents a unique example of sponge radiation in an ancient lake. Lubomirskiidae sponges dominate by biomass other components of the littoral benthos and also inhabit the sublittoral and abyssal zones of Lake Baikal (Efremova 2001). The systematics of

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Lubomirskiidae have yet to be completed, as indicated by both morphological and molecular data (Efremova 2001, 2004; Itskovich et al. 2008, 2013a, 2015).

Recent molecular analyses based on the internal transcribed spacer (ITS) region, COXI, silicatein, and intergenic spacer region mtDNA sequences did not support the current morphology-based freshwater sponge taxonomy (Itskovich et al. 2008, 2013a, 2013b, 2015; Harcet et al. 2010; Erpenbeck et al. 2011). This finding indicates that the taxonomy of freshwater sponges needs urgent revision.

The ITS region is one of the most variable parts of the genome and is suitable for analyses of closely related species (Coleman and Vacquier 2002; Coleman 2007). ITS have been successfully used for phylogenetic reconstructions in freshwater sponges (Addis and Peterson 2005; Itskovich et al. 2008, 2013a, 2015; Harcet et al. 2010; Erpenbeck et al. 2011); however, data about intraspecific variability of ITS in sponges are scarce. ITS analysis did not support monophyly of existing genera and several species within Lubomirskiidae (Itskovich et al. 2008, 2015), but the intraspecific variability of ITS in Baikalian sponges has not been studied.

The aim of this work was to study interspecific and intraspecific variability of ITS of rDNA in the freshwater sponges of 2 families, Lubomirskiidae and Spongillidae, to assess the utility of using ITS sequences for differentiating species, as well as to study the evolution of sponges in Lake Baikal.

Study site

Samples of Spongillidae were collected in small lakes near Irkutsk city (52°23′49.1″N 104°01′19.0″E and 52°27′46.2″N 103°56′54.3″E). Samples of Lubomirskiidae were collected at different sites of northern, middle, and eastern Baikal during expeditions conducted in 1997–2013.

Methods

Sponge samples were frozen in liquid nitrogen for molecular analysis and fixed in 70% ethanol for morphological examination. Spicule and skeleton preparation were performed as previously described (Efremova 2001) and examined using an Olympus CX22 microscope. The shape and consistency of sponges, skeletal characteristics, the form and size of spicules, and their variability in each sample were analyzed. Spicule sizes were determined by results of measurements of 50 spicules within each sample.

Total genomic DNA extraction was performed using the RIBO-sorb RNA/DNA extraction kit (InterLabService, Russia). For amplification of the ~950 bp of the ITS region, primers were selected according to sequences of freshwater sponges from Genbank. Forward and reverse primers (ITS-Its–F1: GTA GGT GAA CCT GCG GAA GGA; ITS-Its–R1: GTT GGT TTC TTT TCC TCC GCT; Itskovich et al. 2015) are nested in 28S and 18S, respectively, and amplified ITS1, 5.8S rDNA, and ITS2.

Polymerase chain reaction (PCR) amplifications of ITS1 and ITS2 were performed on a DNA Engine Dyad thermal cycler (Bio-Rad, USA) using the 5*ScreenMix (Evrogen). The cycle parameters were initial denaturation at 94 °C for 120 s, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 120 s, followed by a final extension of 8 min at 72 °C. Each PCR reaction was purified by electrophoresis in 0.8% agarose gels and eluted by freezing and thawing. Sequencing of both strands of each PCR product was carried out by Macrogen (Korea) using a BIG DYE 3.1 terminator mix on an ABI 377 Sequencer. Chromatograms were analyzed by BioEdit 5.09 (Hall 1999). All sequences have been deposited to GenBank (http://www.ncbi.nlm.nih.gov) with the accession numbers KX572150–KX572210. The electropherograms were manually inspected for potential intragenomic polymorphisms.

The assignment of the sequences obtained from Porifera was performed using the BLAST software program (http://www.ncbi.nlm.nih.gov/blast/). Sequences were initially aligned using ClustalW 1.7 (Thompson et al. 1994) under default parameters including all available sequences of ITS1 and ITS2 of freshwater sponges available from GenBank, with mandatory manual correction. ITS1 and ITS2 sequences were aligned according to secondary structures. Ambiguously aligned regions were excluded as described in Itskovich et al. (2008), resulting in a data matrix of 608 characters.

Phylogenetic trees were constructed using the maximum likelihood (ML) method and Bayesian inference (BI), as implemented in MEGA 5 (Tamura et al. 2011) and MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). Genetic distances in pairwise comparisons between all analyzed sequences were calculated according to Kimura's 2-parameter model. For the ML analysis, the K2P+G model was the best fitting model. The robustness of the ML trees was estimated by bootstrap percentages (Felsenstein 1985) using 500 replicates with heuristic search and stepwise addition starting trees, and by posterior probabilities for BI trees. Bayesian analyses on nucleotide sequences were run with a parallel version of MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). Each Bayesian analysis comprised at least 2 simultaneous runs of 8 Metropolis-coupled Markov chains at the default temperature (0.2 °C) under the most general model (GTR+G+I) because over-parameterization does not negatively affect Bayesian analyses (Huelsenbeck and Rannala 2004). Analyses were terminated after the chains converged significantly, indicated by the average standard deviation of split frequencies <0.01. *Trochospongilla latouchiana* (Spongillidae) and *Ephydatia muelleri* (Spongillidae) were used as outgroups

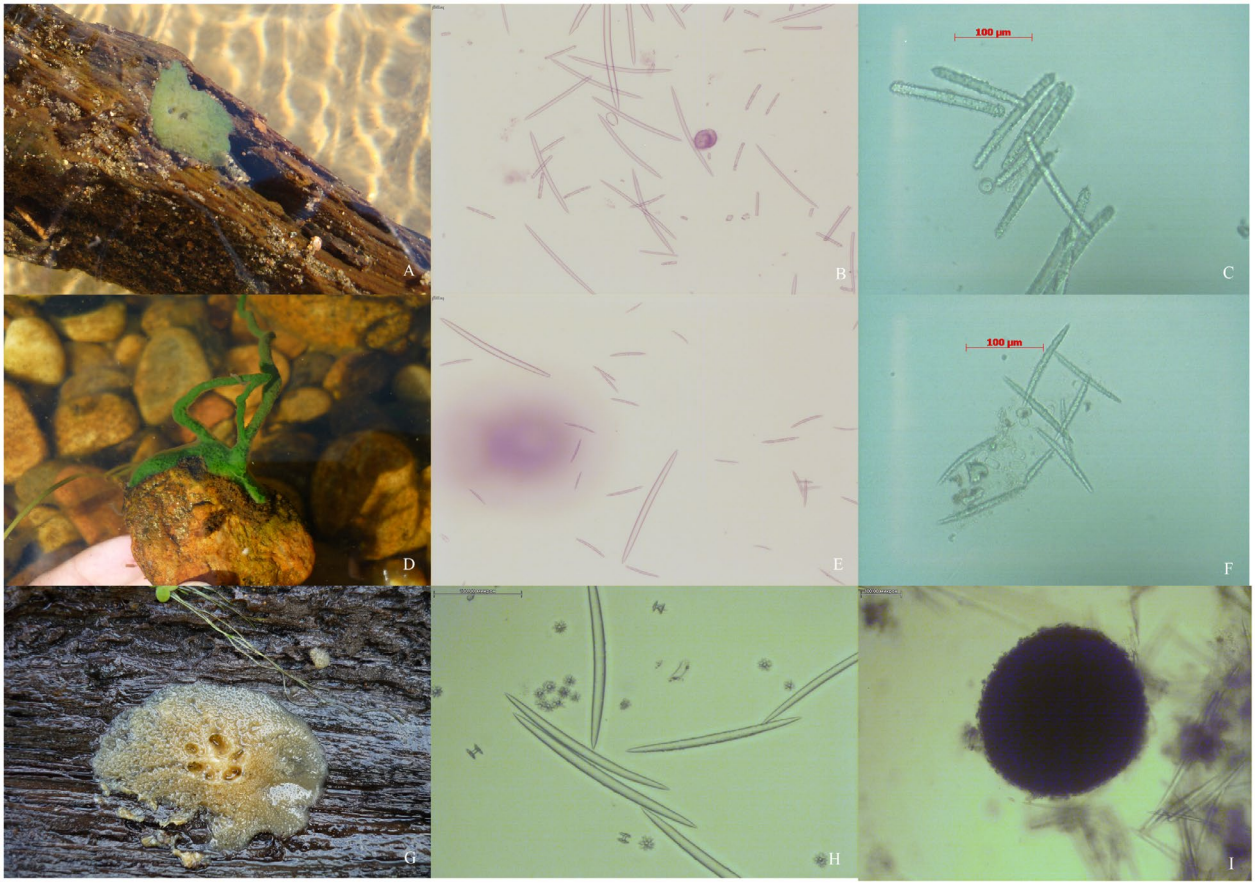

Figure 1. *Eunapius fragilis*: (a) sponge body, (b) megascleres, (c) gemmoscleres; *Spongilla lacustris*: (d) sponge body, (e) megascleres, (f) microscleres; *Ephydatia muelleri*: (g) sponge body, (h) megascleres and gemmoscleres, (i) gemmule.

because their early branching among the Spongillina has been shown in earlier phylogenetic reconstructions (Itskovich et al. 2008).

Results

Spongillidae samples collected from Siberian lakes possess oxea of 90–350 × 2–18 µm covered with small spines, and some samples have microscleres and gemmules. Based on the form and size of gemmosleres, microsleres, and megascleres, samples were identified as *Ephydatia muellery*, *E. fluviatilis*, *Spongilla lacusrtis*, or *Eunapius fragilis* (Fig. 1).

Analyses of spicule morphology revealed that all samples of Lubomirskiidae from Lake Baikal possess oxea of 190–260 × 12–18 µm covered with small spines, but microscleres and gemmules were absent. Microscopy helped identify and classify the samples into 7 species of 3 lubomirskiid genera: *Baikalospongia intermedia*, *B. bacillifera*, *B. martinsoni*, *Lubomirskia baicalensis*, *L. fusifera*, *L. abietina*, and *Rezinkovia echinata* (Fig. 2).

ITS sequences of 7 species of Lubomirskiidae and 4 species of Spongillidae were obtained, varying in length from 889 to 946 nucleotides. All sequences were unique, and differences included substitutions and indels in the ITS1 and ITS2 . A BLAST analysis revealed that the obtained sequences are similar to several Lubomirskiidae species and to the spongillids *E. fluviatilis*, *E. muellery*, *S. lacustris*, and *Eunapius* sp.

Spongillidae have shorter ITS sequences than Lubomirskiidae, so to better align the obtained samples and the available GenBank sequences, sequences of Spongillidae were aligned separately from Lubomirskiidae. After 608 bp alignment, 144 characters were available for phylogenetic analyses. Phylogenetic reconstructions obtained with ML and BI had similar topologies (Fig. 3). All samples identified by morphology as *E. muellery* formed a strongly supported monophyletic clade with a sample of *E. muellery* from Japan (ML 99%, BI 1.0). All *E. fluviatilis* samples also formed a robust clade, including samples from Japan, Israel, and Estonia (ML 97%, BI 1.0). Analyzed samples of *S. lacustris* also form a monophyletic clade with samples from Japan (ML 99%, BI 1.0). Sequence of *Eunapius fragilis* formed a clade with a *Eunapius* species from Japan (ML 99%, BI 1.0). Therefore, newly obtained sequences of *E. fluviatilis*, *E. muellery*, and *S. lacustris*

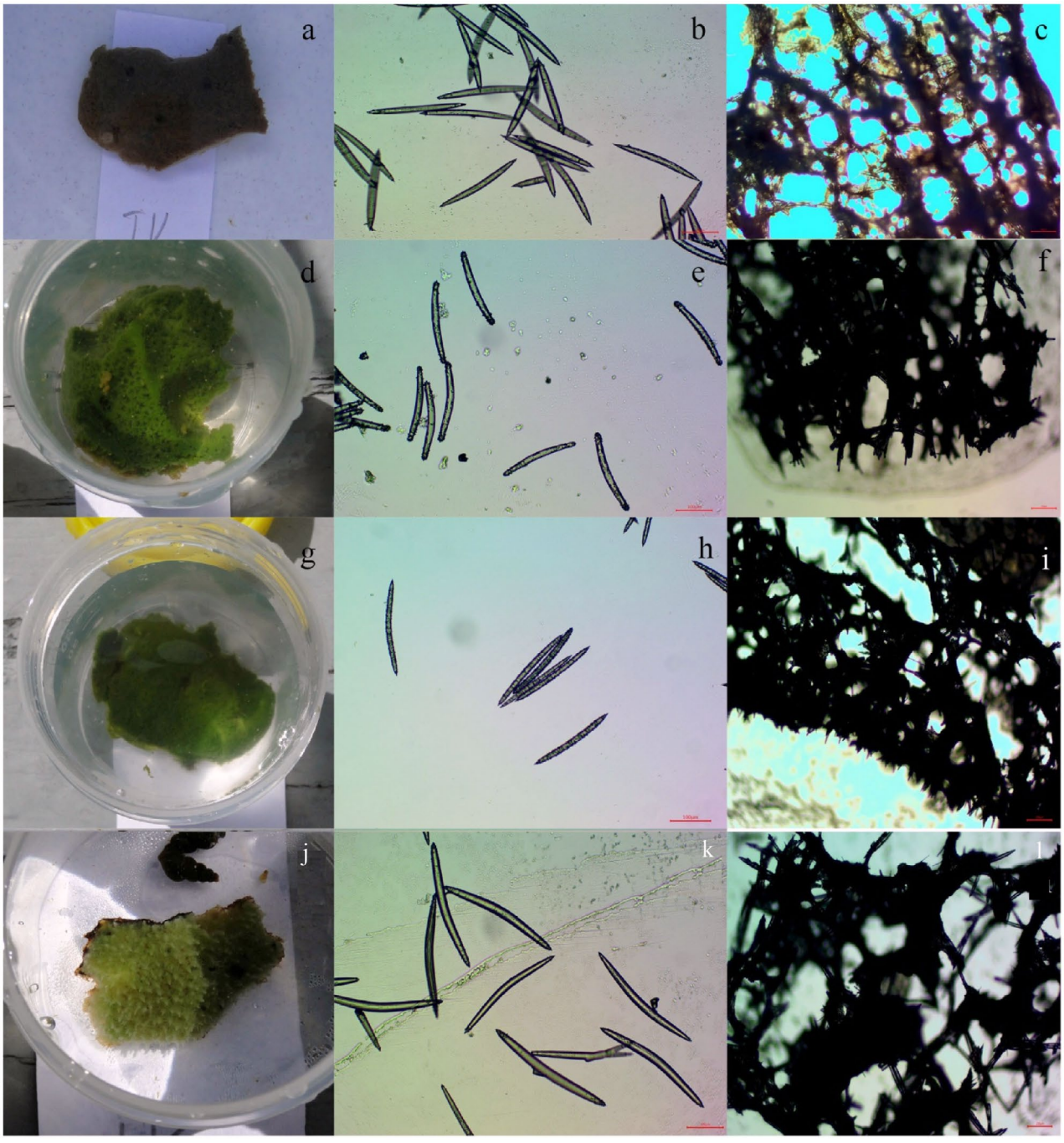

Figure 2. Sponge body, microscopy of megascleres, and the skeleton, respectively, of (a–c) *Baikalospongia recta*; (d–f) *B. martinsoni*; (g–i) *B. intermedia*; and (j–l) *Rezinkovia echinata*.

from East Siberia support monophyly of this species, which is consistent with previous analyses (Itskovich et al. 2008). The level of intraspecies variability of ITS1 and ITS2 was 0–0.5% for *E. muelleri*, 0–1.7% for *E. fluviatilis*, 0.1–0.4% for *S. lacustris*, and 0–2.4% for the *Eunapius* clade. Interspecific variability was significantly larger than the variability within species. Intragenomic differences obtained by Karlep et al. (2013), according to our analyses, were also lower than the interspecific variability.

For better resolution of the relationships within the Lubomirskiidae family, only lubomirskiid sequences were included in the analyses. The 608 bp alignment resulted in 64 characters available for phylogenetic analyses. Relationships within the Lubomirskiidae remain largely undifferentiated (Fig. 4). Samples from different lake basins did not form monophyletic groups. Within Lubomirskiidae were several poorly supported monophyletic groups, including samples of different genera

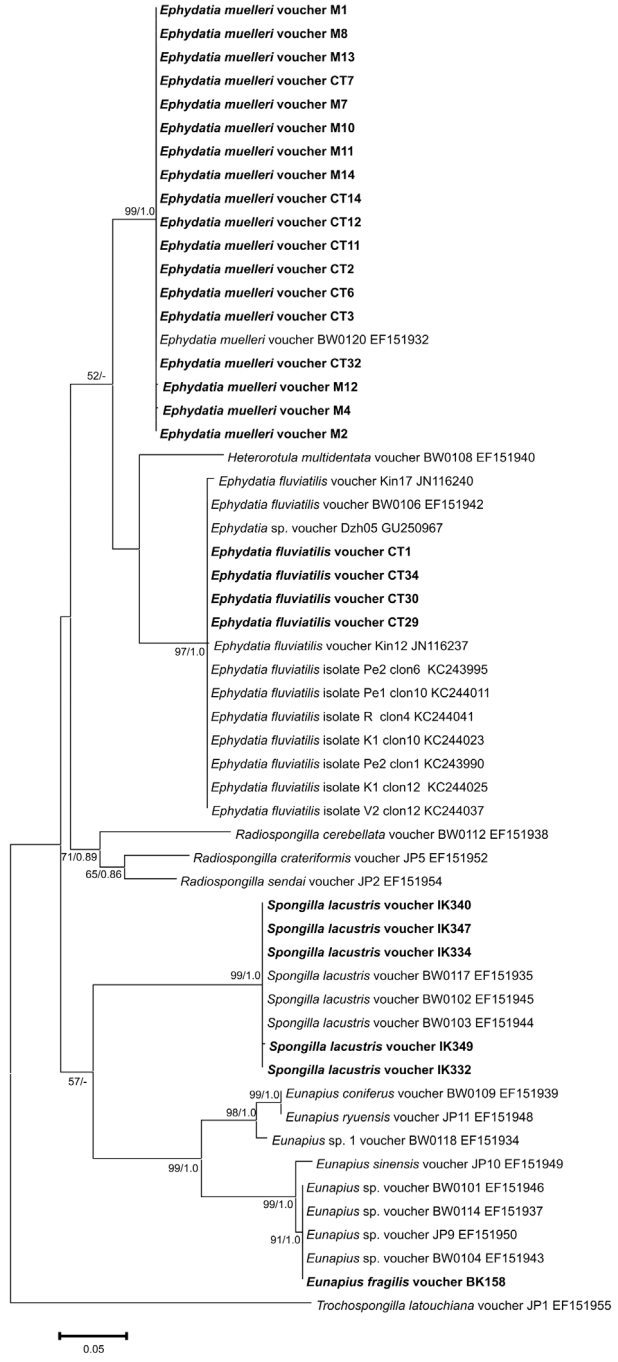

Figure 3. Maximum likelihood phylogenetic tree based on comparisons of 608 bp of ITS1 and ITS2 sequences of Spongillidae. Nodes are characterized by bootstrap percentages (ML; bp > 50%) followed by Bayesian posterior probabilities (PP > 0.85). *Trochospongilla latouchiana* (Spongillidae; GenBank EF151955) were used as outgroups. Taxon names of sequences obtained in this study are indicated in bold. Scale bar denotes substitutions per site.

and species. Results indicated that ITS analyses did not support monophyly of the lubomirskiid genera and species, which is consistent with Itskovich et al. (2008, 2013a, 2015).

Discussion

Sponge systematics have been traditionally based on skeletal traits (spicules and spongin fibres), but spicule complexity, especially in non-gemmulating freshwater sponge genera, is limited. Shape and size of spicules can also vary in different ecological conditions (Poirrier 1974). Sponges are ecologically significant because of their role as biofiltrators and because they serve as the substrate for other organisms, making it important to study their biodiversity. We are, for the first time, studying genetic variability of Spongillidae in Siberia.

Taxonomy of the genera *Spongilla*, *Ephydatia*, and *Eunapius* need revision based on molecular data. More than 150 nominal species were ascribed to the genus *Spongilla*, but they were transferred to other genera, and at present 7 valid species of *Spongilla* have been described (Mancony and Pronzato 2002). *Spongilla lacustris* is a highly variable light-positive species that may contribute significantly to the primary production of small lentic habitats (Frost 1978). Our data support monophyly of *S. lacustris* and a wide area of geographic distribution.

Eunapius Gray, 1867, is also cosmopolitan and includes 13 other species (Mancony and Pronzato 2002). Molecular analyses of sponges from Lake Tanganyika revealed separate *Eunapius* lineages (Erpenbeck et al. 2011). Three genetic markers (COI, 18S rRNA, ITS2) exclude *Eunapius subterraneus* from the genus *Eunapius* and indicate that gemmular traits are not as universally informative as was previously thought (Harcet et al. 2010). Therefore, molecular characterization of additional *Eunapius* samples is important to determine the radiation of *Eunapius*. Our data support monophyly of *Eunapius*, except for *E. subterraneus*. Monophyly or nonmonophyly of *E. fragilis* could not be verified because a sequence was obtained from only one sample.

Ephydatia is a truly cosmopolitan genus with great morphological variability depending on environmental conditions. *Cortispongilla baroisi*, which differs from *Ephydatia* by having larger spicules, was previously shown to be a synonym of *E. fluviatilis* (Itskovich et al. 2013a). The high morphological variability of *Ephydatia* encourages reassessment of species diversity in ancient lakes with special habitat conditions. We obtained the molecular data for *E. fluviatilis* and *E. muellery* from Siberia in addition to the available data from Japan, Israel, and Estonia, and these data support monophyly of these species. Analysis of endemic species that can also be ecomorphs of *Ephydatia* is promising.

We are, for the first time, studying genetic variability of Spongillidae from Siberia. Our findings revealed that ITS1 and ITS2 sequences could be an appropriate tool for phylogenetic study and species identification of Spongillidae, which is consistent with previous studies (Itskovich et al.

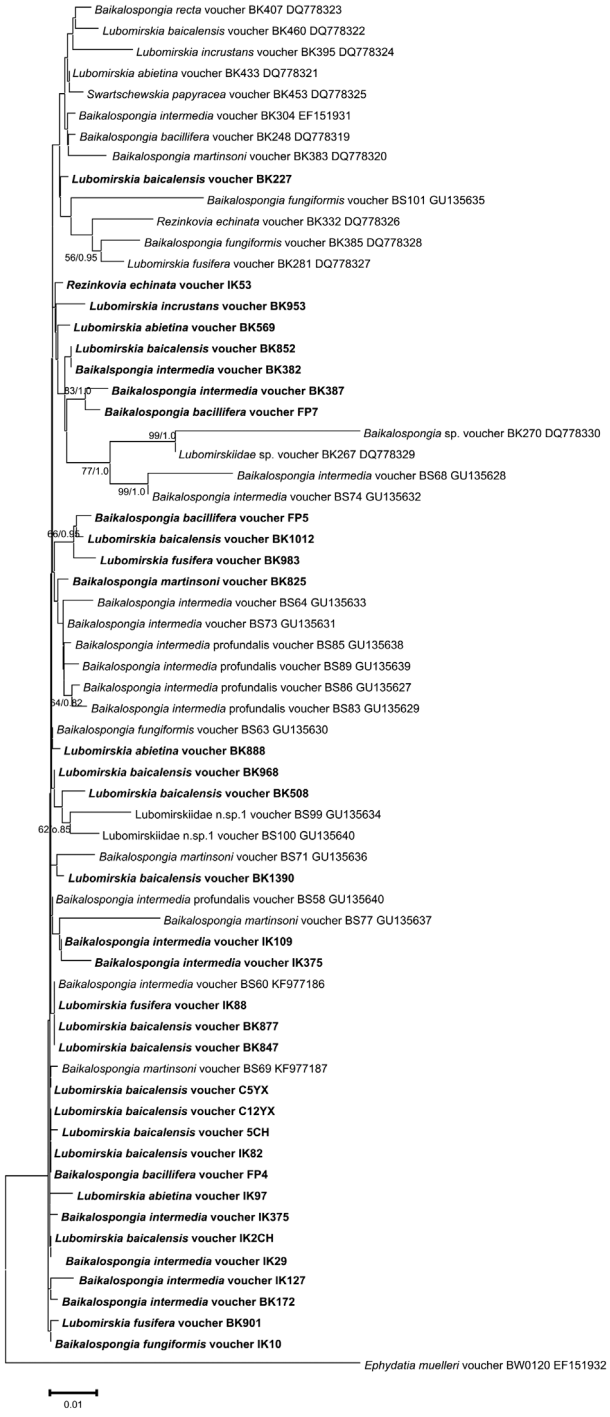

Figure 4. Maximum likelihood phylogenetic tree based on comparisons of 608 bp of ITS1 and ITS2 sequences of Lubomirskiidae. Nodes are characterized by bootstrap percentages (bp > 50%) and Bayesian posterior probabilities (PP > 0.85): ML/BI. *Ephydtia muelleri* (Spongillidae; GenBank EF151932) was used as an outgroup. Taxon names of sequences obtained in this study are indicated in bold. Scale bar denotes substitutions per site.

2013a). Several species for which multiples samples of ITS sequences were available from GenBank and obtained for this study were found to be monophyletic. Intraspecific polymorphisms were much lower than interspecific variability among the species of Spongillidae, despite the analyzed samples originating from geographically distant locations (Siberia, Germany, Japan, Italy). Within species with recent divergence or species complex of the marine sponge genus *Cliona*, interspecific variation of ITS1 and ITS2 sequences overlapped with intraspecific variation, suggesting either incomplete lineage sorting or extensive gene flow (Escobar et al. 2012). Our study confirmed reciprocal monophyly of *E. fluviatilis*, *E. muellery*, and *S. lacustris* and also the adequacy of using ITS as barcoding sequences for DNA taxonomy of Spongillina. Estimating the number of sponge species in ancient lakes is important for understanding the patterns of their evolution as well as for ecological monitoring.

Our results support the monophyly of Lubomirskiidae and agree with previous analyses (Itskovich et al. 2008, 2015). Previously, monophyly of Lubomirskiidae was not supported by CO1 and 18S data because of the high conservatism of these markers (Meixner et al. 2007). It is interesting that compared to Lake Baikal, endemic sponges in Lake Tanganyika have a polyphyletic origin (Erpenbeck et al. 2011). Such deviations in evolution of endemic species flocks in Lake Tanganyika and Lake Baikal were indicated also for ostracods and are most likely related to the different limnological regimes of the 2 lakes (Schön and Martens 2004). Formation of the oxygenated abyss around 5–6 MA ago (Lukin 1986) might have caused radiation of sponge species in Lake Baikal. At that time, the common ancestor of all Lubomirskiidae had already colonized the lake and gave rise to radiation at great depths. This finding is supported by data on explosive speciation among Baikalian benthic organisms at about 3.8–2 MA and is correlated in time with the beginning of the Neobaikalian stage of the Baikal Rift formation (Sherbakov 1999). Another option is that several introductions of sponges may have occurred in Baikal, but after several periods of glaciations that took place in the Baikal region since 4 MA (Mats 1993), only one radiation survived to the present day.

ITS, silicatein gene, and intergenic region of mtDNA analysis did not support monophyly of existing genera and several species within Lubomirskiidae (Itskovich et al. 2008, 2013a, 2015), but the intraspecific variability of ITS in Baikalian sponges is nearly unstudied. The results of this study confirm that molecular data do not support the existing taxonomy of Lubomirskiidae at the genera level. Moreover, several species were not recovered as monophyletic. Current problems with species delimitation in Lubomirskiidae are probably due to their relatively recent divergence time (<5 MA; Itskovich 2005; Maikova et al. 2014). Such discrepancies between molecular and morphological data may be a result of incomplete species divergence among these recently diverged species.

The spongillid species analyzed in this study are cosmopolitan, and their ability to produce gemmules helped them spread throughout inland waters (Mancony and Pronzato 2008). Unlike Spongillidae, Lubomirskiidae lack the ability to gemmulate, which prevents their spread via gemmules and led to the dominance of sexual reproduction. Environmental factors affecting the speciation in sponges of Lake Baikal are not obvious. In our phylogenetic reconstructions, samples from northern, middle, and eastern Baikal did not form separate clades, and neither did shallow- nor deep-water Lubomirskiidae (Itskovich et al. 2015). This result may be associated with incomplete species divergence and indicates that species delimitation is difficult in the case of rapid species radiation within Lubomirskiidae. Absence of asexual reproduction resulted in various mechanisms of speciation in Spongillidae and Lubomirskiidae, which may be the key to understanding the differences in the evolution of cosmopolitan species and endemic species in ancient lakes.

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Disclosure statement

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