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The Utilityof 28S rDNA for Barcoding of Freshwater Sponges (Porifera, Spongillida)

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Abstract: Sponges (Porifera, Spongillida) make up the bulk of the benthic biomass in Lake Baikal and are represented by the family Lubomirskiidae, a collection of endemic species, and several species of the cosmopolitan family Spongillidae. We conducted an analysis of the D3 domain of the 28S rDNA of 16 freshwater sponge species. Based on molecular data, we were able to identify all of the collected Spongillidae specimens whose identification was difficult due to the lack of gemmules. Phylogenetic trees have shown that *Ephydatia muelleri*, *Spongilla lacustris*, and *Eunapius fragilis* formed monophyletic clades, and the D3 domain of the 28S rDNA can be used for their DNA barcoding. For the Baikal sponges, the use of this marker is important since the gemmule-less Spongillidae and Lubomirskiidae are, in some cases, indistinguishable from each other in morphology. The 28S rDNA has been shown to be useful for family and species-level identification of freshwater sponges within the Spongillida.

Keywords: Porifera; DNA barcoding; Lake Baikal; freshwater sponges; 28S rDNA

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1. Introduction

Baikal sponges (Porifera, Spongillida) make up the bulk of the benthic biomass in Lake Baikal [1] and play an important ecological role as biofiltersand are home to a pro- and eukaryotic symbiotic community. The mass disease of Baikal sponges, which began in 2010 and continues to the present, reinforces the importance of assessing and conserving their biodiversity [2–4]. The available evidence that the symbionts of the Baikal sponges are the producers of biologically active substances also makes it relevant to develop Lubomirskiidae taxonomy and species identification [5]. Sponges in Lake Baikal are represented by two families. Lubomirskiidae (about 15 species) is an endemic bouquet of species formed during rapid speciation [6,7]. Species identification of Lubomirskiidae is difficult both on the basis of morphological and molecular methods. To date, all molecular analyses based on the ITS region, COXI, silicatein, and intergenic spacer region mtDNA sequences have not supported the current morphology-based taxonomy of Lubomirskiidae [8–11]. Genomic data can help to solve this problem. The first phylogenomic studies of Baikal sponges based on transcriptomic data showed the monophyly of the genus *Lubomirskia* and an earlier divergence of *Swartschewskia papyracea* from a common ancestor [12,13]. However, genomic sequencing of large numbers of samples is expensive and requires, first of all, the selection of the most interesting targets for phylogenomics, namely genetically divergent samples.

The second sponge family inhabiting Lake Baikal, Spongillidae, is a cosmopolitan one, with over 150 species found worldwide [14]. In Lake Baikal, five species of Spongillidae [6,15] have been described on the basis of morphology. Molecular studies based on ITS1 and ITS2 spacers of rDNA confirmed the existence of four species of Spongillidae in Lake Baikal, but the presence of *Trochospongilla* in Baikal has not been confirmed with molecular data [16]. The complexity of the species identification of Spongillidae in Lake Baikal is due to the fact that under constant living conditions at a considerable depth, they have lost the ability to form gemmules. The structure of gemmules and gemmoscleres is

the most important diagnostic feature [6,15]. Moreover, Lubomirskiidae and gemmule-less Spongillidae are not unambiguously verified by morphological characters [17].

The most used molecular markers for species identification and phylogeny analysis of freshwater sponges are the COX1 gene fragment and the ITS1 and ITS2 spacers of rDNA. Both of these markers have their advantages and disadvantages. For the Folmer fragment of the COX1 gene, there are a sufficient number of freshwater sponge sequences in the Genbank, but it has been shown that it is a conservative marker, and many sponge species have identical sequences [8,18–21]. ITS1 and ITS2 spacers of rDNA have good resolution at the species and genus levels, but their use is difficult in the higher-level taxonomic groupings [9,11,16,21,22]. The ITS1 and ITS2 spacers worked well at the species level in Spongillidae because the species studied formed monophyletic clusters. However, in closely related Lubomirskiidae, ITS spacers have revealed polyphyly of genera and species, probably due to the recent divergence or influence of intragenomic polymorphism [9,16,23]. Therefore, the search for new molecular markers remains relevant for freshwater sponges.

Baikal organisms, including sponges, are a model for studying sympatric speciation [12,24]. 28S rDNA has been successfully used to analyze species bouquets in other speciation hotspots. Thus, molecular characterization of the *Hyalella* (Crustacea: Amphipoda) using 28S rDNA sequences revealed the presence of five evolutionarily distant lineages within Lake Titicaca [25]. Analysis of 28S rDNA, together with other markers, revealed an undescribed species of the Antarctic gastropod family Velutinidae [26]. 28S rDNA evolves faster than 18S rDNA, and there are both conserved and variable regions present [27]. 28S rDNA has been successfully used to analyze the phylogeny of marine sponges individually and in conjunction with other genes. 28S rRNA gene data would help to resolve poriferan phylogenetic relationships at the sub-ordinal level [28]. The D3-D5 region of this gene was successfully employed to examine relationships between haplosclerid species [29–31] and resulted in the re-classification of some Heteroscleromorpha [32,33]. However, to the present day, this marker has essentiallyremained unused for freshwater sponges [34,35].

To remedy this, we performed the first analysis of 28S rDNA in Baikal sponges to both identify collected specimens and assess the potential of 28S rDNA as a tool for barcoding freshwater sponges.

2. Materials and Methods

Samples of sponges were collected at two collection points in Central Baikal: MaloeMore strait, Kurma village (53°11′496″ N 106.59′752″ E) and MaloeMore strait, Zama village (53°28′275″ N 107°32′022″ E) during expeditions performed in 2022. For all samples, in vivo images were captured (Figure 1). Sponge samples were used for DNA extraction immediately after their collection and fixed in 70% ethanol for morphological examination. Some of the samples were keptalive and kept in aquariums for further molecular analysis. Species identification of specimens was based on the morphological analysis of the skeleton and spicules using an Olympus CX22 (Olympus Corporation, Tokyo, Japan) light microscope and a Philips SEM 525M (Royal Philips Electronics Inc., Amsterdam, The Netherlands) scanning electron microscope. The shape and consistency of sponges, skeletal characteristics, the shape and size of spicules, and their variability in each sample were analyzed (Figure 2). Total genomic DNA extraction was performed using an ExtractDna Blood&Cells kit (Evrogen, Moscow, Russia). The D3 domain of the 28S rDNA was amplified as previously described [34,35]. Polymerase chain reaction (PCR) amplification of ITS1 and ITS2 wasperformed on a DNA Engine Dyad thermal cycler (Bio-Rad, Hercules, CA, USA) using the Taq DNA polymerase (Evrogen). The cycle parameters were initial denaturation at 94 °C for 120 s, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 60 s, followed by a final extension of 8 min at 72 °C. Each PCR reaction was purified by electrophoresis in 0.8% agarose gels, cut from the gel and eluted by freezing and thawing. Sanger sequencing of both strands of each PCR product was carried out with Novogene Co., Ltd., China using the original primers. Chromatograms were ana-

lyzed using BioEdit 5.0 (Bioedit Company, Manchester, UK) 9 [36]. All sequences have been deposited with GenBank (http://www.ncbi.nlm.nih.gov) (accessed on 1 December 2022) with accession numbers OP558490-OP558521. Assessment of the sequences obtained was performed using the BLAST software program (http://www.ncbi.nlm.nih.gov/blast/) (accessed on 1 December 2022). Sequences were initially aligned using ClustalW 1.7 [37] under default parameters, including all available sequences of the 28S rRNA gene of freshwater sponges available from GenBank, with mandatory manual correction. Genetic distances in pairwise comparisons between analyzed sequences were calculated according to Kimura's 2-parameter model. Phylogenetic trees were constructed using the maximum likelihood (ML) method as implemented in MEGA 5 [38]. For the ML analysis, the K2+G model was found to bethe bestfit. The robustness of the ML trees was estimated by bootstrap percentages [39] using 500 replicates with heuristic search and stepwise addition starting trees. Bayesian analyses on nucleotide sequences were run with a parallel version of MrBayes 3.1.2 [40]. Each Bayesian analysis comprised at least two simultaneous runs of eight Metropolis coupled Markov chainsunder the most general model (GTR + G + I) because overparameterization does not negatively affect Bayesian analyses [41]. Analyses were terminated after the chains converged significantly, indicated by the average standard deviation of split frequencies < 0.01. The robustness of the Bayesian trees was estimated using posterior probabilities. *Trochospongilla horrid* Weltner, 1893 (Spongillidae) was used as the outgroup for 28S rDNA sequences becausean early branching position of this genus among Spongillida has been shown in previous phylogenetic reconstructions [9,42].

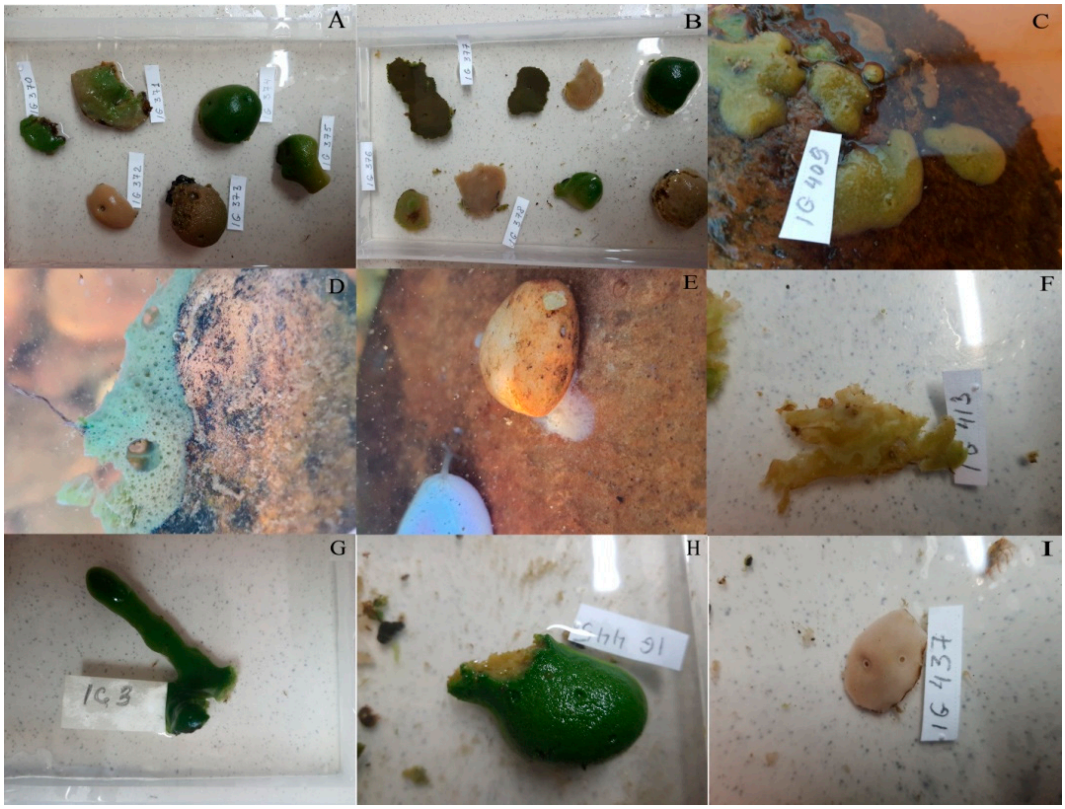

Figure 1. (**A**,**B**) In vivo and (**C**) in situ photos of Lubomirskiidae from Lake Baikal; (**D**,**E**) In situ and (**F**) in vivo photos of Spongillidae from Lake Baikal; (**G**) *L. baikalensis*; (**H**) *B. bacillifera*; (**I**) *S. papyracea*.

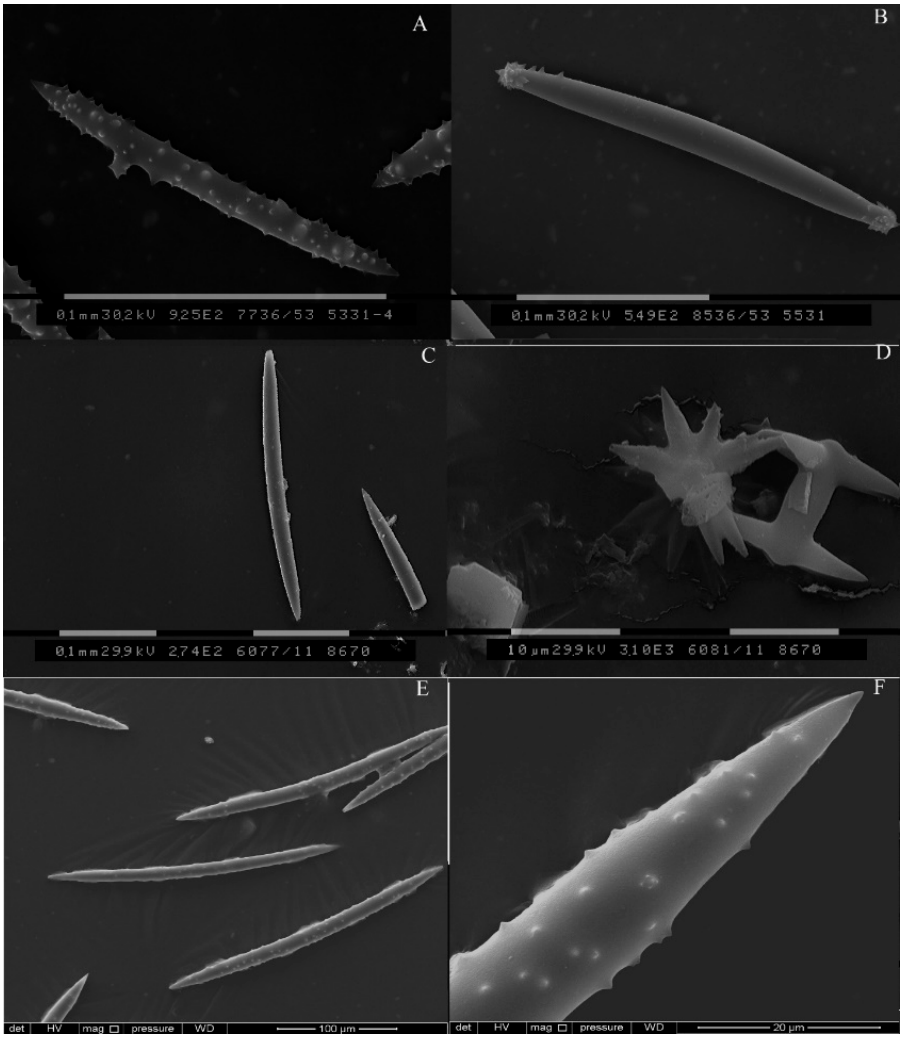

Figure 2. (**A**) megascleres of *L. baikalensis*; (**B**) megascleres of *B. bacillifera*; (**C**) megascleres of *E. muelleri*; (**D**) gemmuloscleres of *E. muelleri*; (**E**,**F**) megascleres of *Spongillida* sp.

3. Results and Discussion

Based on morphological analysis, the collected samples were identified as *Lubomirskia baikalensis*, *L. fusifera*, *L. abietina*, *Baikalospongia intermedia*, *B. bacillifera*, *B. recta*, *Swartschewskia papyracea*, *Spongillalacustris*, *Eunapius* sp., *Ephydatia* sp. (Figures 1 and 2). Species identification of Spongillidae using morphology is difficult because it is based on the structure of gemmules, gemmuloscleres, and microscleres [14]. In the summer season, in nearby water bodies and shallow bays, and also throughout the year in Lake Baikal itself, due to the constancy of habitat conditions, spongillids do not form gemmules [6,15]. While Spongilla also have microscleres, Ephydatia only have megascleres, an oxeas similar to that of the shallow-water Lubomirskiidae. Some samples had intermediate morphological characteristics to determine their belonging to the family and species (Figures 1 and 2). Their differential characteristics were long thin spicules, which are characteristic of both the Lubomirskiidae and some Spongillidae of open Baikal [6,17]. Additionally, some of the specimens assigned to Lubomirskiidae could not be identified up to a species due to a wide intraspecific morphological variability, as noted earlier [15,17].

A fragment of approximately 340 nucleotides corresponding to the D3 domain of sponge 28S rDNA was obtained for 31 specimens of Lubomirskiidae and 6 specimens of Spongillidae. BLAST analysis revealed that the obtained sequences were similar to several Spongillidae species (data not shown). The sequences were aligned with available GenBank sequences of Spongillida (Figure 3) and, after exclusion of regions that are not

unambiguously alignable, resulted in 278 bp alignment in which 14 characters were variable and 8 characters were available for phylogenetic analyses.

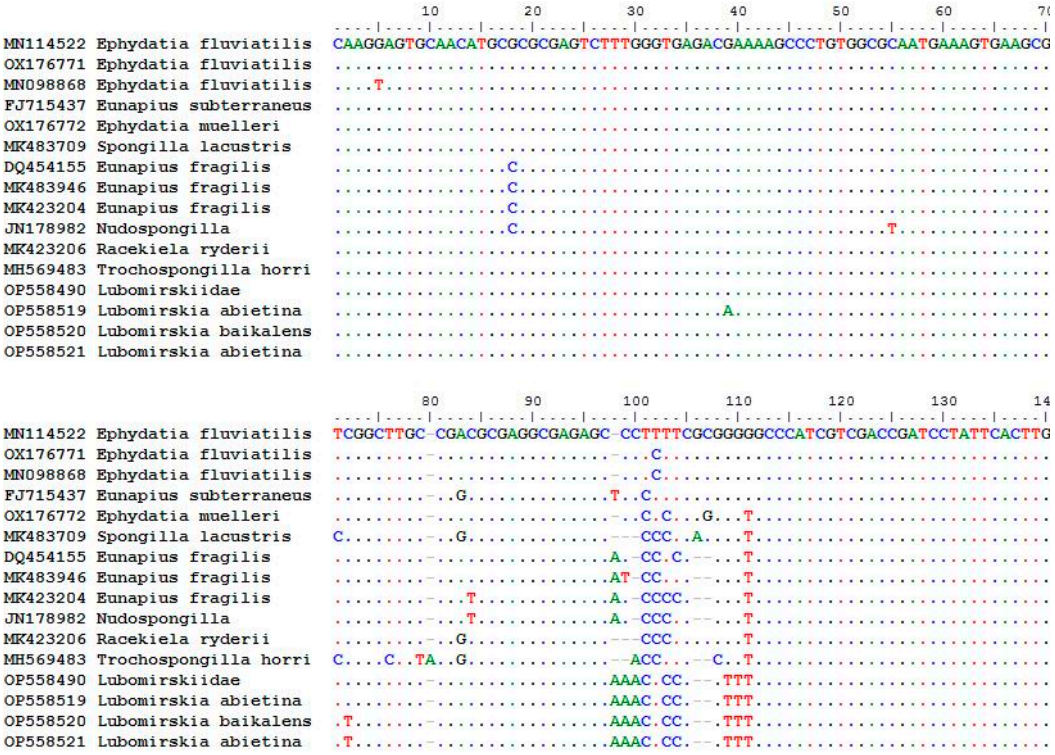

Figure 3. Fragment of alignment of 28S rDNA D3 region sequences of Spongillida before exclusion of regions that are not unambiguously alignable. Dots indicate identity with the first sequence, and dashes are inferred insertion–deletion.

All Spongillidae sequences assigned morphologically and by molecular analyses to different species had nucleotide substitutions. The level of intraspecies variability of the 28S rDNA fragment was 0.3–0.7% for *E. fluviatilis* and 0.7–1.4% for the *Eunapius* clade. Sequences of 7 analyzed samples of *E. muelleri* collected from Lake Baikal, Italy, Germany, and Estonia were identical to each other. Additionally, 5 sequences of *S. lacustris* samples collected from Lake Baikal, Germany, Serbia, and Estonia were identical to each other. Interspecific variability (overall mean distance) within Spongillidae was 2% and was larger than the variability within species. Of the 31 analyzed samples of Lubomirskiidae, which belong to 7 species, 28 samples have identical sequences: *Baikalospongia recta* (OP558498, OP558490), *Baikalospongia intermedia* (OP558494-OP558497, OP558501, OP558502, OP558504, OP558506, OP558508, OP558515), *Baikalospongia bacillifera* (OP558492, OP558505, OP558507), *Lubomirskia baikalensis* (OP558509, OP558510, OP558514), *Lubomirskia abietina,* (OP558516, OP558518), *Swartschewskia papyracea* (OP558491, OP558517), and 3 samples had substitutions (3 transitions): *Lubomirskia abietina* (OP558519, OP558521) and *Lubomirskia baikalensis* (OP558520).

Phylogenetic trees showed that sequences of *Ephydatia muelleri* (7 sequences), *Spongilla lacustris* (5 sequences), and *Eunapius fragillis* (4 sequences) specimens formed monophyletic clades with high posterior probabilities (Figure 4). All analyzed species of Spongillidae differ in this gene fragment by nucleotide substitutions and deletion–insertions (Figure 3). It is noteworthy that samples of each species from geographically remote areas were included in the analysis. Also noteworthy is the fact of greater genetic divergence of *Trochospongilla horida*, which is also confirmed by the analysis of other genetic markers [9,11,16,21,22]. Therefore, sequence alignment and our phylogenetic analyses identified 28S rDNA as the suitable barcoding marker for Spongillidae.

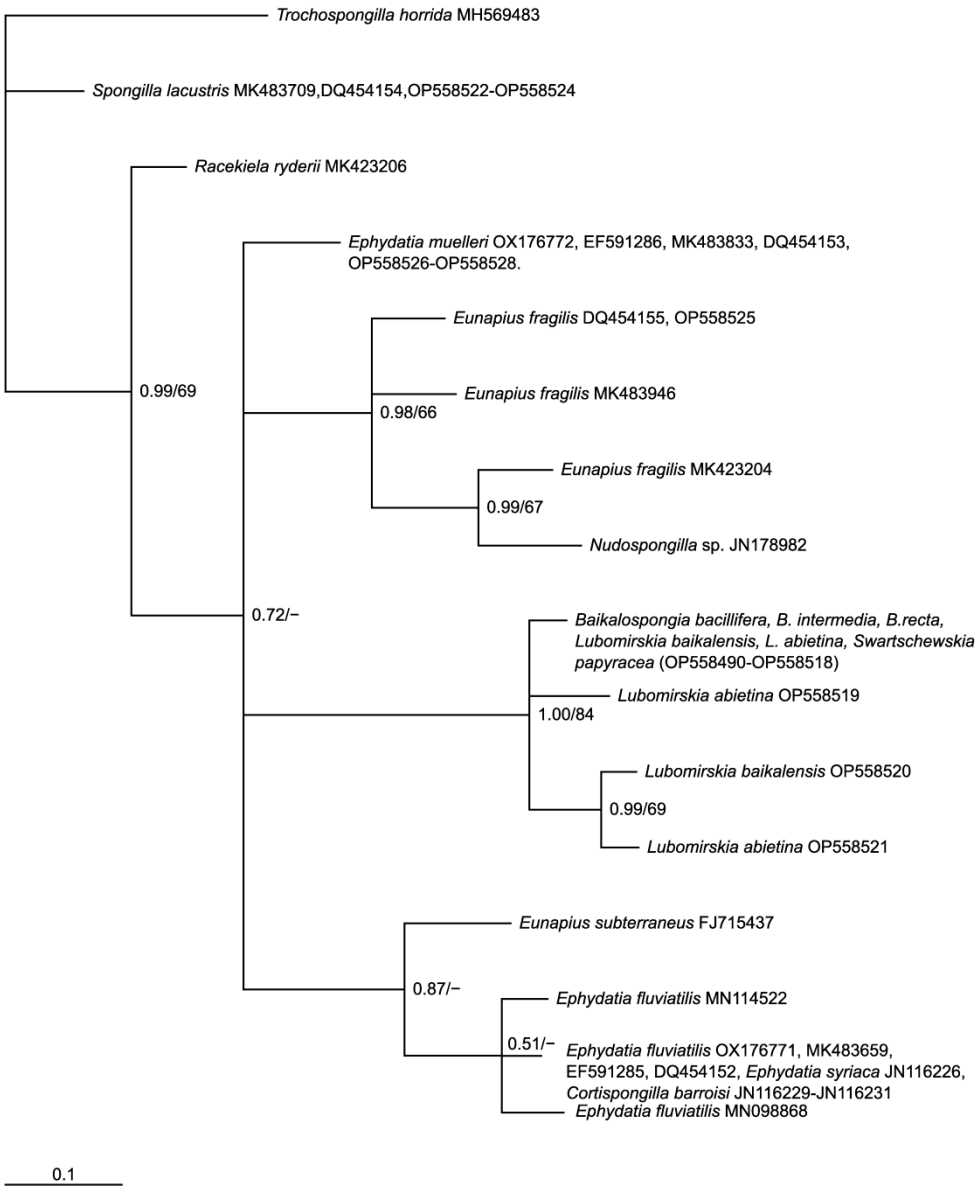

Figure 4. Bayesian phylogenetic tree based on comparisons of 287 bp of 28S rDNA sequences of Spongillida. Nodes are characterized by Bayesian posterior probabilities (PP > 0.50) followed by bootstrap percentages (ML; bp > 50%). *Trochospongilla horrida* (Spongillidae; GenBank MH569483) was used as the outgroup. Scale bar indicates substitutions per site at unit distance.

Three genetic markers (COI, 18S rRNA, ITS2) exclude *Eunapius subterraneus* from the genus *Eunapius* [43]. 28S rDNA analyses support this finding and revealed close relationships of *E. subterraneus* with *Ephydatia* (Figure 3). The non-monophyletic nature of *Ephydatia* on the 28S rDNA tree supports the previous opinion about the paraphyly of this genus [11,42]. Since gemmule traits are not as universally informative as was previously thought [43], the analysis of several markers with different evolutionary rates is strictly necessary when analyzing spongillid phylogeny.

The 28S rDNA analysis supports the identity of *Cortispongilla barroisi* and *E. fluviatilis*, which is also supported by the analysis of other markers [44]. Analysis of the ITS2 spacer sequence of the museum holotypes of *C. barroisi* also confirmed the closeness of *C. barroisi* and *E. fluviatilis* [11,45]. All of this points to the artificial nature of the family Malawispongiidae and the usefulness of 28S rDNA for revising the taxonomy of Spongillidae at the family level in the future.

Previously, ITS1 and ITS2 spacers of rDNA were shown to be the only well-functioning markers for species identification of Spongillidae; however, alignment problems and the impact of intragenomic variability have been discussed [9,11,23]. Furthermore, ITS spacers have not shown good resolution above the genus level [11,21,22]. Therefore, the search for additional molecular markers for the analysis of sponge phylogeny is relevant. The advantage of short sequences has been shown in the analysis of old museum holotypes [11]. During long-term storage, DNA is partially destroyed and long fragments cannot be amplified and sequenced. Thus, the possibility of amplifying short fragments (100 bp) for the analysis of samples more than 100 years old has been shown to be useful [11,39]. We assume that 28S rDNA fragments couldbe used for the same purpose when a sufficient base of sequences of different species is accumulated.

For the first time, we conducted an analysis of 28S rDNA in freshwater sponges, including an analysis of 16 species, and have shown the promise of this marker forspecies identification. It is also possible that the use of a longer 28S rDNA fragment, such as the D3-D5 region, will increase its resolution for closely-related sponge species.

On the phylogenetic tree based on 28S rDNA, Lubomirskiidae are monophyletic. This corresponds to previously obtained results and confirms the single introduction of this endemic family into Lake Baikal [8,9,17]. Species delimitation within Lubomirskiidae is difficult due to the high morphological variability of Baikal sponges. The presence of substitutions in the 28S rDNA fragment in several samples indicates genetic divergence and the possibility of the existence of new, undescribed species. Two of the three divergent specimens had morphology similar to the already described species of Lubomirskiidae. The absence of morphological differences in the presence of genetic divergence was previously noted for the deep-water sponges of Lake Baikal and may be a sign of cryptic speciation [17,46]. However, numerous Lubomirskiidae specimens identified as different species have identical sequences, which may indicate a lack of species boundaries. The substitutions in the conservative region may indicate a real genetic divergence, which should be refined with the help of other, more variable regions and genomics data. Preliminary identification of these samples based on inexpensive short-sequence analysis is a necessary step prior to obtaining genomic data.

The mass disease that has recently affected different species of Baikal sponges [2–4] reinforces the importance of developing our understanding of their taxonomy for the assessment and conservation of biodiversity. To date, only analysis of genomic and transcriptomic data has recovered resolved phylogenies at the genus level within the Lubomirskiidae [12,13]. Large-scale ddRAD analyzes of a large number of samples, including those genetically divergent in the 28S rDNA fragment, allowing us to specify the species composition of Lubomirskiidae, are in progress.

4. Conclusions

We have shown the applicability of 28S rDNA for the barcoding of Spongillidae. We also showed the usefulness of this marker for determining where samples belong within the families of gemmule-less Spongillidae and Lubomirskiidae found in Lake Baikal. It is likely 28S rDNA analysis will be useful for analyzing the phylogenetic relationships of taxa above the genus level within Spongillida after the accumulation of 28S rDNA data for other freshwater sponge species.

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Data Availability Statement: Baikalian sponge specimens are stored in the collection of the Limnological Institute SB RAS, Irkutsk, Russia. The 28S D3 sequences were deposited in the NCBI GenBank (accession numbers OP558490-OP558521).

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Timoshkin, O.A. Lake Baikal: Fauna diversity, problems of its immiscibility and origin, ecology and exotic communities. In *Index of Animal Species Inhabiting Lake Baikal and Its Catchment Area*; Timoshkin, O.A., Ed.; Nauka: Lake Baikal, Russia, 2001; Volume 1, pp. 74–116.
2. Timoshkin, O.A.; Samsonov, D.P.; Yamamuro, M.; Moore, M.V.; Belykh, O.I.; Malnik, V.V.; Sakirko, M.V.; Shirokaya, A.A.; Bondarenko, N.A.; Domysheva, V.M.; et al. Rapid ecological change in the coastal zone of Lake Baikal (East Siberia): Is the site of the world's greatest freshwater biodiversity in danger? *J. Great Lakes Res.* **2016**, *42*, 487–497. [CrossRef]
3. Khanaev, I.V.; Kravtsova, L.S.; Maikova, O.; Bukshuk, N.; Sakirko, M.V.; Kulakova, N.V.; Butina, T.V.; Nebesnykh, I.A.; Belikov, S.I. Current state of the sponge fauna (Porifera: Lubomirskiidae) of Lake Baikal: Sponge disease and the problem of conservation of diversity. *J. Great Lakes Res.* **2018**, *44*, 77–85. [CrossRef]
4. Itskovich, V.B.; Shigarova, A.M.; Glyzina, O.Y.; Kaluzhnaya, O.V.; Borovskii, G.B. Heat shock protein 70 (Hsp70) response to elevated temperatures in the endemic Baikal sponge *Lubomirskiabaicalensis*. *Ecol. Indic.* **2018**, *88*, 1–7. [CrossRef]
5. Kaluzhnaya, O.V.; Itskovich, V.B. Features of diversity of polyketide synthase genes in the community of freshwater sponge *Baikalospongia fungiformis*. *Russ. J. Genet.* **2022**, *58*, 336–346. [CrossRef]
6. Efremova, S.M. Sponges (Porifera). In *Index of Animal Species Inhabiting Lake Baikal and Its Catchment Area*; Timoshkin, O.A., Ed.; Nauka: Lake Baikal, Russia, 2001; Volume 1, pp. 182–192.
7. Efremova, S.M. New genus and new species of sponges from family LubomirskiidaeRezvoj, 1936. In *Index of Animal Species Inhabiting Lake Baikal and Its Catchment Area*; Timoshkin, O.A., Ed.; Nauka: Lake Baikal, Russia, 2004; Volume 1, pp. 1261–1278.
8. Meixner, M.J.; Luter, C.; Eckert, C.; Itskovich, V.; Janussen, D.; von Rintelen, T.; Bohne, A.V.; Meixner, J.M.; Hess, W.R. Phylogenetic analysis of freshwater sponges provide evidence for endemism and radiation in ancient lakes. *Mol. Phyl. Evol.* **2007**, *45*, 875–886. [CrossRef] [PubMed]
9. Itskovich, V.; Gontcharov, A.; Masuda, Y.; Nohno, T.; Belikov, S.; Efremova, S.; Meixner, M.; Janussen, D. Ribosomal ITS sequences allow resolution of freshwater sponge phylogeny with alignments guided by secondary structure prediction. *J. Mol. Evol.* **2008**, *67*, 608–620. [CrossRef]
10. Itskovich, V.B.; Kaluzhnaya, O.V.; Belikov, S.I. Investigation of nuclear and mitochondrial DNA polymorphism in closely related species of endemic Baikal sponges. *Russ. J. Genet.* **2013**, *49*, 966–974. [CrossRef]
11. Erpenbeck, D.; Steiner, M.; Schuster, A.; Genner, M.J.; Manconi, R.; Pronzato, R.; Ruthensteiner, B.; van den Spiegel, D.; van Soest, R.W.M.; Worheide, G. Minimalist barcodes for sponges: A case study classifying African freshwater Spongillida. *Genome* **2019**, *62*, 1–10. [CrossRef]
12. Kenny, N.J.; Plese, B.; Riesgo, A.; Itskovich, V.B. Symbiosis, selection and novelty: Freshwater adaptation in the unique sponges of Lake Baikal. *Mol. Biol. Evol.* **2019**, *36*, 2462–2480. [CrossRef]
13. Kenny, N.J.; Itskovich, V.B. Phylogenomic inference of the interrelationships of Lake Baikal sponges. *Syst. Biodivers.* **2021**, *19*, 209–217. [CrossRef]
14. Manconi, R.; Pronzato, R. Suborder Spongillinasubord. nov.: Freshwater sponges. In *Systema Porifera. A Guide to the Classification of Sponges*; Hooper, J.N.A., Van Soest, R.W.M., Eds.; Kluwer Academic/Plenum Publishers: New York, NY, USA; Boston, MA, USA; Dordrecht, The Netherland; London, UK; Moscow, Russia, 2002; pp. 921–1020. [CrossRef]
15. Masuda, Y. Studies on the taxonomy and distribution of freshwater sponges in Lake Baikal. In *Biosilica in Evolution, Morphogenesis and Nanobiotechnology*; Müller, W.E.G., Grachev, M.A., Eds.; Springer: Berlin/Heidelberg, Germany, 2009; pp. 81–110. [CrossRef]
16. Itskovich, V.; Glyzina, O.; Kaluzhnaya, O. Intraspecific and interspecific sequence variability in the ITS region of the rDNA of freshwater sponges of Lake Baikal and East Siberia. *Inland Water* **2017**, *7*, 259–266. [CrossRef]
17. Itskovich, V.B.; Kaluzhnaya, O.V.; Veynberg, E.; Erpenbeck, D. Endemic Lake Baikal sponges from deep water. 1: Potential cryptic speciation and discovery of living species known only from fossils. *Zootaxa* **2015**, *3990*, 123–137. [CrossRef] [PubMed]
18. Schroder, H.C.; Efremova, S.M.; Itskovich, V.B.; Belikov, S.; Masuda, Y.; Krasko, A.; Muller, I.M.; M€uller, W.E.G. Molecular phylogeny of the freshwater sponges in Lake Baikal. *J. Zool. Syst. Evol. Res.* **2003**, *41*, 80–86. [CrossRef]
19. Erpenbeck, D.; Weier, T.; De Voogd, N.J.; Worheide, G.; Sutcliffe, P.; Todd, J.A.; Michel, E. Insights into the evolution of freshwater sponges (Porifera: Demospongiae: Spongillina): Barcoding and phylogenetic data from Lake Tanganyika endemics indicate multiple invasions and unsettle existing taxonomy. *Mol. Phyl. Evol.* **2011**, *61*, 231–236. [CrossRef] [PubMed]
20. Yakhnenko, A.S.; Itskovich, V.B. Analysis of the variability of mtDNA in closely related species of Baikalian sponges for the development of new barcoding markers. *Limnology* **2020**, *21*, 49–57. [CrossRef]

21. Sokolova, A.M.; Palatov, D.M.; Itskovich, V.B. Genetic analysis confirms the freshwater origin of the endemic Caspian sponges (Demospongiae, Spongillida, Metschnikowiidae). *ZooKeys* **2020**, *915*, 1–16. [CrossRef]
22. Carballo, J.L.; Cruz-Barraza, J.A.; Yanez, B.; Gomez, P. Taxonomy and molecular systematic position of freshwater genus Racekiela (Porifera: Spongillida) with the description of a new species from North-west Mexico. *Syst. Biodivers.* **2018**, *16*, 160–170. [CrossRef]
23. Itskovich, V.B. Intragenomic variation of rDNA internal transcribed spacers in the endemic Baikal sponge *Lubomirskiabaicalensis*: Implications for Porifera barcoding. *J. Great Lakes Res.* **2020**, *46*, 62–66. [CrossRef]
24. Naumenko, S.A.; Logacheva, M.D.; Popova, N.V.; Klepikova, A.V.; Penin, A.A.; Bazykin, G.A.; Etingova, A.E.; Mugue, N.S.; Kondrashov, A.S.; Yampolsky, L.Y. Transcriptome-based phylogeny of endemic Lake Baikal amphipod species flock: Fast speciation accompanied by frequent episodes of positive selection. *Mol. Ecol.* **2017**, *26*, 536–553. [CrossRef]
25. Adamowicz, S.J.; Marinone, M.C.; Menu-Marque, S.; Martin, J.W.; Allen, D.C.; Pyle, M.N.; De los Ríos, P.; Sobel, C.N.; Ibáñez, C.; Pinto, J.; et al. The *Hyalella* (Crustacea: Amphipoda) species cloud of the ancient Lake Titicaca originated from multiple colonizations. *Mol. Phyl. Evol.* **2018**, *125*, 232–242. [CrossRef]
26. Fassio, G.; Modica, M.V.; Alvaro, M.C.; Buge, B.; Salvi, D.; Oliverio, M.; Schiaparelli, S. An Antarctic flock under the Thorson's rule: Diversity and larval development of Antarctic Velutinidae (Mollusca: Gastropoda). *Mol. Phyl. Evol.* **2019**, *132*, 1–13. [CrossRef]
27. Mindell, D.; Honeycutt, R. Ribosomal RNA in Vertebrates: Evolution and phylogenetic applications. *Annu. Rev. Ecol. Syst.* **1990**, *21*, 541–566. [CrossRef]
28. McInerney, J.O.; Adams, C.I.; Kelly, M. Phylogenetic resolution potential of 18S and 28S rRNA genes within the lithistid Astrophorida. *Mem. Queensl. Mus.* **1999**, *44*, 343–351.
29. McCormack, G.P.; Erpenbeck, D.; van Soest, R.W.M. Major discrepancy between phylogenetic hypotheses based on molecular and morphological criteria within the Order Haplosclerida (Phylum Porifera: Class Demospongiae). *J. Zool. Syst. Evol. Res.* **2002**, *40*, 237–240. [CrossRef]
30. Redmond, N.E.; Raleigh, J.; van Soest, R.W.; Kelly, M.; Travers, S.A.; Bradshaw, B.; Vartia, S.; Stephens, K.M.; McCormack, G.P. Phylogenetic relationships of the marine Haplosclerida (Phylum Porifera) employing ribosomal (28S rRNA) and mitochondrial (*cox1, nad1*) gene sequence data. *PLoS ONE* **2011**, *6*, e24344. [CrossRef] [PubMed]
31. Morrow, C.; Allcock, L.A.; Mccormack, G. A new species of *Hymeraphia* Bowerbank, 1864 (Axinellida: Raspailiidae) from a deep-water canyon southwest off Ireland. *Zootaxa* **2018**, *4466*, 61–68. [CrossRef]
32. Erpenbeck, D.; Breeuwer, J.A.J.; van Soest, R.W.M. Implications from a 28S rRNA gene fragment for the phylogenetic relationships of halichondrid sponges (Porifera: Demospongiae). *J. Zool. Syst. Evol. Res.* **2005**, *43*, 93–99. [CrossRef]
33. Erpenbeck, D.; Hooper, J.N.A.; List-Armitage, S.E.; Degnan, B.M.; Wörheide, G.; van Soest, R.W.M. Affinities of the family Sollasellidae (Porifera, Demospongiae). II. Molecular evidence. *Contrib. Zool.* **2007**, *76*, 95–102. [CrossRef]
34. Lopp, A.; Reintamm, T.; Vallmann, K.; Päri, M.; Mikli, V.; Richelle-Maurer, E.; Kelve, M. Molecular identificati on characterization and distribution of freshwater sponges (Porifera: Spongillidae) in Estonia. Fundam. *Appl. Limnol. (Archiv für Hydrobiologie)* **2007**, *168*, 93–103. [CrossRef]
35. Roovere, T.; Lopp, A.; Reintamm, T.; Kuusksalu, A.; Richelle-Maurer, E.; Kelve, M. Freshwater sponges in Estonia: Genetic and morphological identification. *Proc. Estonian Acad. Sci. Biol. Ecol.* **2006**, *55*, 216–227. [CrossRef]
36. Hall, T.A. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/ NT. *Nucl. Acids Symp. Ser.* **1999**, *41*, 95–98. [CrossRef]
37. Thompson, J.D.; Higgins, D.G.; Gibson, T.J. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucl. Acids Res.* **1994**, *22*, 4673–4680. [CrossRef]
38. Tamura, K.; Peterson, D.; Peterson, N.; Stecher, G.; Nei, M.; Kumar, S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **2011**, *28*, 2731–2739. [CrossRef] [PubMed]
39. Felsenstein, J. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **1985**, *39*, 783–791. [CrossRef]
40. Ronquist, F.; Huelsenbeck, J.P. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **2003**, *19*, 1572–1574. [CrossRef] [PubMed]
41. Huelsenbeck, J.P.; Rannala, B. Frequentist properties of Bayesian posterior probabilities of phylogenetic trees under simple and complex substitution models. *Syst. Biol.* **2004**, *53*, 904–913. [CrossRef] [PubMed]
42. Addis, J.S.; Peterson, K.J. Phylogenetic relationships of freshwater sponges (Porifera, Spongillina) inferred from analyses of 18S rDNA, COI mtDNA, and ITS2 rDNA sequences. *Zool. Scr.* **2005**, *34*, 549–557. [CrossRef]
43. Harcet, M.; Bilandžija, H.; Bruvo-Mađarić, B.; Ćetković, H. Taxonomic position of *Eunapiussubterraneus* (Porifera, Spongillidae) inferred from molecular data—A revised classification needed? *Mol. Phyl. Evol.* **2010**, *54*, 1021–1027. [CrossRef]
44. Itskovich, V.; Kaluzhnaya, O.; Ostrovsky, I.; McCormack, G. The number of endemic species of freshwater sponges (Malawispongiidae; Spongillina; Porifera) from Lake Kinneret is overestimated. *J. Zool. Syst. Evol. Res.* **2013**, *51*, 252–257. [CrossRef]
45. Erpenbeck, D.; Galitz, A.; Wörheide, G.; Albrecht, C.; Pronzato, R.; Manconi, R. Having the balls to colonize—The *Ephydatiafluviatilis* group and the origin of (ancient) lake "endemic" sponge lineages. *J. Great Lakes Res.* **2020**, *46*, 1140–1145. [CrossRef]
46. Itskovich, V.; Kaluzhnaya, O.; Veynberg, Y.; Erpenbeck, D. Endemic Lake Baikal sponges from deep water. 2: Taxonomy and bathymetric distribution. *Zootaxa* **2017**, *4236*, 335–342. [CrossRef] [PubMed]