Phylogenetic analysis of freshwater sponges provide evidence for endemism and radiation in ancient lakes

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Abstract

Morphologic and phylogenetic analysis of freshwater sponges endemic to lakes in Central Sulawesi, Siberia and South-East Europe is presented. We also analyzed several cosmopolitan sponge species from Eurasia and North America and included sponge sequences from public databases. In agreement with previous reports (Addis, J.S., Peterson, K.J., 2005. Phylogenetic relationships of freshwater sponges (Porifera, Spongillina) inferred from analyses of 18S rDNA, COI mtDNA, and ITS2 rDNA sequences. Zool. Scr. 34, 549–557), the metaniid sponge Corvomeyenia sp. was the most deeply branching species within a monophyletic lineage of the suborder Spongillina. Pachydictyum globosum (Malawispongiidae) and Nudospongilla vasta (Spongillidae), two morphologically quite distinct species from Sulawesi were found in a joint clade with Trochospongilla (Spongillidae) rendering Trochospongilla paraphyletic. Furthermore, Ochridaspongia sp., another Malawispongiidae, clustered far away from that clade, together with Ephydatia fluviatilis, making the latter family polyphyletic. The Lubomirskiidae endemic to Lake Baikal, Lubomirskia abietina, Baikalospongia bacillifera, B. intermedia, and Swartschewskia papyracea formed a well-supported clade that was most closely linked to the genus Ephydatia (99.9% identity over a total length of 2169 concatenated nucleotide positions). Our study indicates the frequent and independent origin of sponge species endemic to different freshwater ecosystems from a few cosmopolitan founder species. The highly specific primer sets newly developed here facilitate work on the molecular phylogeny and DNA barcoding of sponges.

Keywords: Demospongiae; Lake Baikal; Mitochondrial cytochrome oxidase subunit I; Molecular taxonomy; Porifera; Sulawesi lakes; Translational exceptions

1. Introduction

The majority of extant Porifera are restricted to the marine environment, but a few taxa within the Demospongiae live in freshwater habitats. All freshwater sponges were combined into a new haplosclerid suborder Spongillina, comprising six extant families, Spongillidae, Lubomirskiidae, Malawispongiidae, Metaniidae, Metschnikowiidae and Potamolepidae (Manconi and Pronzato, 2002). The Spongillidae show a worldwide distribution, whereas the other five families are endemic or are geographically restricted. Sequence analyses of 18S and 28S rDNA, the mitochondrial cytochrome oxidase I gene (COI), and ITS2 rDNA have been proven useful to infer their phylogenetic relationships (Addis and Peterson, 2005; Borchiellini et al., 2004; Nichols, 2005). Based on such data, the monophyletic origin of freshwater sponges has been suggested (Addis and Peterson, 2005), which is in contrast to very recent work suggesting reassessment of the order

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Haplosclerida (Redmond et al., 2007). In fact, several analyses have pointed out that freshwater sponges are not true Haplosclerida: According to Borchelliini et al. (2004), the Spongillina form a monophylum outside the marine Haplosclerida (represented by Halichondria mediterranea), whereas in Nichols (2005) the Haplosclerina are a well-supported clade with exclusion of the Spongillina. More molecular analyses such as those introduced by Borchelliini et al. (2004) and Nichols (2005) would be helpful to illuminate the phylogenetic relationships between freshwater and marine haplosclerids and to other demosponges in more detail.

The origin of Spongillidae has been dated to a time between 183 and 141 MYa by using both minimum evolution (ME) and maximum likelihood (ML) molecular clocks (Peterson and Butterfield, 2005). The phylogenetic relationships between and within the freshwater sponge families are not fully resolved yet; however, it has been suggested that the genus Ephydatia is paraphyletic and that species belonging to the families Lubomirskiidae, Mtschinkowiidae and Malawispongiidae may have arisen from species of that genus (Addis and Peterson, 2005).

To further elucidate the relationships among freshwater porifera, a DNA-based phylogenetic analysis of species endemic to geographically and geologically distinct freshwater lakes could be informative. For this purpose, sponges were sampled in old tectonic lakes on Sulawesi (Indonesia), in lakes Baikal and Dzhegetai-Kul (SE Siberia) as well as in Lake Ohrid (Macedonia). This collection was complemented by common freshwater sponges from lakes and rivers in Brandenburg and Mecklenburg (NE Germany) and from river Rhine (W Germany) and by freshwater sponge-derived sequences from public databases.

Lake Poso and Lake Matano in Central Sulawesi are oligotrophic and in many geological aspects very similar. Based on their endemic faunal elements, Sarasin and Sarasin (1905) assumed a Late Miocene to Pliocene age; however, according to new fossil track data, Late Tertiary to Early Pleistocene (~2 MYa) appears more likely (Bellier et al., 2006). The epilimnion temperature ranges from 27 to 31 °C in both lakes and varies only slightly from 27 °C in the hypolimnion (Haffner et al., 2001). The largely endemic sponge fauna of these lakes was neglected for almost a century. The most recent report from 1901 described three species (Weltner, 1901), all without gemmules, the typical asexual reproducing stages for spongillids.

Lake Baikal (Siberia) is the largest and oldest lake in the world since it can be dated back to the early Miocene (Martinson, 1936, 1938). Due to its age and special geological situation without large inlets and only one outlet, a unique fauna and flora developed in Lake Baikal over several geological epochs. It possesses an endemic sponge fauna, together with several ubiquitous species of the family Spongillidae. A typical underwater photo from Lake Baikal is shown in Fig. 1a with “sponge forests” consisting mainly of branching Lubimorisia baikalensis and other incrusting Lubomirskiidae as the dominant megafaunal element. With a biomass of more than 1 kg/m² in some littoral areas (Kozhov, 1963), sponges play a prominent ecological role in the shallow water habitats (6–20 m depths) of Lake Baikal. But sponges are found also in the deeper zones of Baikal and have been collected from large depths, as far as down to 889 m (Rezvoi, 1936). Temperatures of Lake Baikal are low throughout the year: The surface water varies between ~1 and about +10 °C for Winter and Summer, respectively. Below ca. 30 m water depth, the temperature is constant at 4 °C. Lake Dzhegetai-Kul is located about 750 km SW from Lake Baikal on the foot of Tannu-Ola Mountains at an elevation of about 1000 m. The oligotrophic lake has a maximum depth of 17 m and is probably of Holocene age, about 10,000 years ago. The lake is habitat of the non-gemmulating sponge Baikalospongia dzhugatajensis (Rezvoi, 1936). The first mention of sponges from Lake Baikal was by the traveler P.S. Pallas (1771), who described the most prominent littoral species under the name Spongia baikalensis (now Lubomirskia baikalensis). Dybowskii (1880) wrote the first monograph on all baikalian sponges known in the 19th century, which he united into the genus Lubomirskia. In the early 20th century two further genera were described, Baikalospongia Annandale, 1914 and Swartscheswskia Makushok, 1927. A revised system of the sponges from Lake Baikal was published by Rezvoi (1936), who also described Baikalospongia dzhugatajensis, which he attributed to the Lubomirskiidae. Some authors assigned this species to the Spongillidae (Greze and Greze, 1958; Kozhov, 1972), however, in Müller et al. (2006) it was positioned again within the Lubomirskiidae. B. dzhugatajensis shows morphological affinities to Baikalospongia in spicule shape and skeletal architecture; however, until this work no molecular data existed for this species and its phylogenetic affiliation remained unresolved. In a thorough revision of the baikalian sponges, Efremova (2001, 2004) described a new genus, Rezinkovia, and several new species of the Lubomirskiidae. According to Efremova (2001), the following valid genera (and species) are currently known from Lake Baikal: Lubomirskiidae: Lubomirskia (4 sp.), Baikalospongia (4 sp., 1 ssp.), Swartscheswskia (2 ssp.) and Rezinkovia (2 sp.); Spongillidae: Ephydatia (2 ssp.), Spongilla (1 sp.), Eunapius (1 sp.) and Trochospongilla (1 species).

The oligotrophic Lake Ohrd being 286 m is the deepest lake in SE Europe. It is situated on the Macedonian–Albanian border and covers an area of 340 km². The hypolimnion temperature varies only slightly from 6 °C and the surface layer never cools below 3.94 °C (Outcalt and Allen, 1981). Because of the absence of geophysical and sedimentological studies the absolute geological age is still unknown. Spirkovski et al. (2001) speculated that the lake was formed between 4 and 10 MYa ago, but earlier data indicated an age between 2 and 3 MYa (Stankovic, 1960). Lake Ohrd represents a refuge for numerous endemic freshwater organisms, whose close relatives can be found as fossil remains from the Tertiary period (Stankovic,
Five different sponge species were described from this lake (Arndt, 1938; Gilbert and Hadzisce, 1984; Hadzisze, 1953) and one, *Ochridaspongia* sp., is included here. Based on molecular sequence data from nuclear and mitochondrial gene loci, we tested if sponges endemic to these different freshwater ecosystems would share a common or partially common evolutionary history or originated independently from each other from a few cosmopolitan founder species.

2. Materials and methods

2.1. Investigation areas, sampling, and taxonomic characterization

An overview on all species analyzed in this study is given in Table 1. Sponges from Lake Poso and Lake Matano were collected by SCUBA diving during binational German–Indonesian expeditions in 2003 and 2004. The here investigated species from Lake Baikal were collected by SCUBA diving during expeditions in 1997–1999, within a Russian-Japan joint research program to Lake Baikal. A specimen of *Baikalospongia dzhegatajensis* from Lake Dzhegetai-Kul was kindly provided by Dr. Igor Ivanov (Academy of Sciences, Kysyl, Russia). A set of common *Spongillidae* was collected in the lakes Schmaler Luzin (Germany, Mecklenburg, 2003), Flakensee/Kalksee (Germany, Berlin/Brandenburg area, 2004) and in the Lücknitz River (Germany, Berlin/Brandenburg area, 2003). *Trochospongilla horrida* (Weltner, 1901) was collected on the left embankment of Rhine River near Neuburg in November 2005 and kindly provided by Dr. Jochen Gugel (University of Stuttgart, Germany). *Ochridaspongia* sp. was collected in Lake Ohrid, and kindly provided by Dr. Christian Albrecht (University of Giessen, Germany). As outgroups to freshwater sponges, sequences were obtained from the marine sponges *Pseudaxinella reticulata*, *Microciona prolifera* and *Scopalina ruetzleri* (collected and kindly provided by Drs. Klaus Rützler and Wolfgang Sterrer).

All sponges were inspected for possible contamination by invertebrate commensals. Part of each sample was fixed immediately in 70% ethanol for taxonomic identification.
<table>
<thead>
<tr>
<th>Species/sample</th>
<th>Lineage</th>
<th>Origin/reference</th>
<th>COI GenBank Accession No.</th>
<th>18 rRNA GenBank Accession No.</th>
<th>Voucher No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baikalospongia bacillifera</td>
<td>Haplosclerida; Lubomirskiida</td>
<td>Russia, Lake Baikal, W shore, Bolshie Koty, 14–15 m, July 2000</td>
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<td>Baikalospongia dzhagatajensis</td>
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<td>Russia, Tuvinian Autonom Republic, Lake Chagytai, N shore; leg.: Igor Ivanov, 1999</td>
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<td>n.d.</td>
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<td>Baikalospongia intermedia</td>
<td>Haplosclerida; Lubomirskiida</td>
<td>Russia, Lake Baikal, W shore, Bolshie Koty, 14–15 m, July 2000</td>
<td>DQ167168</td>
<td>DQ167154</td>
<td>ZMB Por 12653</td>
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<tr>
<td>Corvomeyenia sp. Addis03–32</td>
<td>Haplosclerida; Metanidae</td>
<td>(Addis and Peterson, 2005)</td>
<td>DQ176781</td>
<td>DQ176774</td>
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<tr>
<td>Ephydatia cooperensis (Peterson and Addis, 2000)</td>
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<td>(Peterson and Addis, 2000)</td>
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<td>AF140354</td>
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<td>Ephydatia sp.</td>
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<td>Germany, Rüdersdorf (nr. Berlin), Lake Kalksee, May 2004</td>
<td>DQ167173</td>
<td>n.d.</td>
<td>ZMB Por 12660</td>
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<td>Ephydatia fluviatilis 1 (Linnaeus, 1759)</td>
<td>Haplosclerida; Spongillida</td>
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<td>AJ843884</td>
<td>AJ705048</td>
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</tr>
<tr>
<td>Ephydatia fluviatilis 2 (Linnaeus, 1759)</td>
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<td>Germany, Rüdersdorf (nr. Berlin), Lake Kalksee, July 2004</td>
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<td>DQ167158</td>
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<td>COI: (Addis and Peterson, 2005), 18S: (Richelle-Maurer et al., 2006)</td>
<td>DQ176777</td>
<td>AY578146</td>
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<td>(Peterson and Addis, 2000)</td>
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<td>DQ167159</td>
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<td>Eunapius fragilis 1 (Leidy, 1851)</td>
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<td>(Peterson and Addis, 2000)</td>
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<td>Eunapius fragilis 2 (Leidy, 1851)</td>
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<td>USA, North Carolina, October 2003</td>
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<td>n.d.</td>
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<td>Eunapius carteri (Bowerbank, 1863)</td>
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<td>Germany, Erkner (nr. Berlin), Channel to Löcknitz River, July 2004</td>
<td>DQ167175</td>
<td>DQ167160</td>
<td>ZMB Por 12661</td>
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<td>Lubomirskia abietina (Swartschewski, 1901)</td>
<td>Haplosclerida; Lubomirskiida</td>
<td>Russia, Lake Baikal Listvianka, 12 m, July 2000</td>
<td>DQ167170</td>
<td>DQ167156</td>
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<td>Lubomirskia baikalensis (Pallas 1771)</td>
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<td>Russia, Lake Baikal, Listvianka, 8 m, July 2000</td>
<td>DQ167169</td>
<td>DQ167155</td>
<td>ZMB Por 12654</td>
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<td>Microciona prolifera (Ellis &amp; Solander, 1786)</td>
<td>Poecilosclerida; Microcionidae</td>
<td>U.S., Massachusetts coast near Woods Hole, 2004</td>
<td>AJ843888</td>
<td>AJ705047</td>
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<td>Nudospongilla vasta 1 (Weltner, 1901)</td>
<td>Haplosclerida; Spongillida</td>
<td>Indonesia, Sulawesi, Lake Matano, S shore, nr. cave entrance, 2003</td>
<td>DQ167179</td>
<td>DQ167165</td>
<td>ZMB Por 12651</td>
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<tr>
<td>Nudospongilla vasta 2 (Weltner, 1901)</td>
<td>Haplosclerida; Spongillida</td>
<td>Indonesia, Sulawesi, Lake Matano, S shore, nr. cave entrance, 2003</td>
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<td>DQ167166</td>
<td>ZMB Por 12652</td>
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<tr>
<td>Ochridaspongia sp.</td>
<td>Haplosclerida; Malawispongiida</td>
<td>Macedonia, Lake Ohrid, Ohrid Bay, 35–15 m, leg.: Christian Albrecht, September 2004</td>
<td>EF025855</td>
<td>n.d.</td>
<td>ZMB Por 12689</td>
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<td>Pachydictyum globosum (Weltner, 1901)</td>
<td>Haplosclerida; Malawispongiida</td>
<td>Indonesia, Sulawesi, Lake Poso, Cape Bancea, 2003</td>
<td>DQ167177</td>
<td>DQ167163</td>
<td>ZMB Por 12649</td>
</tr>
</tbody>
</table>
and another part was frozen in liquid nitrogen for molecular analysis. Species used for molecular genetic approaches were identified according to spicule morphology and skeletal architecture (Fig. 1b–i,k). Samples of all specimens are kept in the Museum of Natural History at Humboldt-University Berlin, Germany.

2.2. Isolation of total genomic DNA

About 0.5 g fresh or ethanol-preserved material was homogenized in a mortar under liquid nitrogen and transferred into 15 ml Falcon tubes using a spatula chilled in liquid nitrogen. 1 ml (v/w) 2·CTAB-buffer (2% CTAB, 100 mM Tris–HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl) was added, vortexed and incubated for 15 min at 65 °C in a water bath. One total volume chloroform/isoamylalcohol (24:1) was added and vortexed for 1 min. For phase separation, samples were centrifuged for 10 min at 2500 g at room temperature (RT). The upper phase was transferred into new tubes, the exact amount checked and 1/5 volume 5·CTAB buffer (5% CTAB, 0.35 M NaCl) added, mixed and incubated at 65 °C for 10 min. Following chloroform extraction as before, 1–1.5 vol 1·CTAB (1% CTAB, 50 mM Tris–HCl pH 8.0, 10 mM EDTA) was given to the upper phase for precipitation of DNA for 1 h at RT. After centrifugation at 2500 g for 20 min, the pellet was resuspended in 1 ml of HS-TE (10 mM Tris–HCl pH 8.0, 1 mM EDTA, 1 M NaCl). In some cases the pellet needed to be incubated at 65 °C for 10 min to achieve complete resuspension. DNA was precipitated from this suspension by 3 vol of 96% ethanol/3 M NaOAc 30:1. Following centrifugation and a 70% ethanol wash step, the pellets were air-dried and finally resuspended in 50 μl of sterile water.

2.3. Oligonucleotide primers, PCR conditions, molecular cloning and sequencing

PCR was performed in a total volume of 50 μl, containing the following: 5 μl 10× reaction buffer (Qiagen), final concentration of 3 mM MgCl₂, 2.5 μl dNTP mix (2.5 mM each), 20 pmole of each primer and 1 U Taq polymerase (Qiagen). PCRs were performed on a PTC-200 thermal cycler (MJ research). The PCR regime for amplification was: 1.5 min at 96 °C, followed by 35 cycles with each 30 s 96 °C; 40 s 53–58 °C; 3 min 72 °C, and a final extension step at 72 °C for 30 min. PCR fragments were either purified directly using Qiaquick columns, or by elution from 1% agarose gels and subsequent column purification. The purified DNA fragments were either cloned into vector pDrive (Qiagen) or sequenced directly with the respective PCR primers driving the sequencing reaction. The primer pair HC02198/ LCO1490 (Table 2, Pop et al., 2003) was used for amplification of a fragment of the mitochondrial cytochrome oxidase subunit I gene (COI) from Spongilla lacustris. Exploiting sequence information from marine sponges, we subsequently modified these primers to be either 3′ truncated by 5 nucleotides (HC02198B), or con-
tain one additional nucleotide at the 5′ end and four at the 3′ end (LCO1490B). However, the frequent and tight association between sponges and other eukaryotic organisms turned out as a particular technical challenge due to the frequent amplification of contaminants. To avoid the contaminants, we developed a more specific primer set, COI-fresh-fw/COI-fresh-rv, taking several unique positions into

3

the reverse primer needed to be modified again, by slightly shifting it along the known COI sequences, reducing specificity through introduction of a fourfold degeneracy, yielding primer COI-sula-rv. In order to have matching physical properties, COI-fw-M was developed which is based on COI-fresh-fw-M. However, for the sponges from Sulawesi, the reverse primer needed to be modified again, by slightly shifting it along the known COI sequences, reducing specificity through introduction of a fourfold degeneracy, yielding primer COI-sula-rv. In order to have matching physical properties, COI-fw-M was developed which is based on COI-fresh-fw-M, but two nucleotides shorter at the 5′ end. As a result we obtained 20 new high quality COI sequences from freshwater sponges and 23 new sequences altogether from all sponges analyzed. The complete list of oligonucleotides is given in Table 2.

For amplification of 18S rDNA, we used the primer pair 18a_mdl/18b_mdl (Medlin et al., 1988). However, only partial sequences were obtained for *Lubomirskia baicalensis* and *Pachydictyum globosum* n. sp. For *Ochridaspongia* sp. it was not possible to obtain rDNA amplicons. Altogether, 15 novel nearly complete and two partial 18S rDNA sequences were obtained. In all cases, both DNA strands were sequenced using standard protocols and the Big Dye Terminator chemistry version 1.1 (Applied Biosystems Inc.) on an Applied Biosystems 373 or 377 automated DNA sequencer. Sequencing data were manually corrected, edited, blasted against Genbank and aligned using ABI software SeqEd, SeqAlign, or Lasergene (DNASTAR). All sequences have been deposited in GenBank, accession numbers are given in Table 1.

2.4. Inference of phylogenetic relationships

A multiple alignment of 18S and COI sequences was performed using ClustalX 1.81 (Thompson et al., 1994). Multiple alignment parameters were 15 for gap opening and 6.66 for gap extension. In case of the mitochondrial COI gene, lengths of the initial PCR fragments differed due to the use of different primer sets for the different species. Therefore, the 28 sequences selected for analysis were between 496 and 681 nt long with an overlap of 486 nt between all of them. The main criterion for selecting outgroups was the availability of a COI as well as of an 18S rDNA sequence. In preliminary analyses we tested several additional COI sequences of different marine sponges belonging to distinct phylogenetic groups (*Suberites domuncula*, *Pros-uberites laughlini*, *Geodia media* and *Haliclona amphioxa*) which we downloaded from GenBank but found that they had no effect on the relationships between the here compared freshwater sponges. In addition, selected freshwater sponge sequences were obtained from Genbank. These included *Ephydatia cooperensis*, *E. muelleri*, *Eunapius fragilis*, *Corvomeyenia* sp. and *Trochospongilla pennsylvanica* of North American origin (Addis and Peterson, 2005; Peterson and Addis, 2000; Peterson and Butterfield, 2005).

The total alignment length of 18S rDNA sequences was 1694 nt. For *Ochridaspongia* sp. it was not possible to get
an 18S sequence since the sample was contaminated by mites (Arachnidae) and except *Lubomirskia baicalensis* and *Pachydictyum globosum* n. ssp. for which we obtained only truncated sequences (1143 bp for *P. globosum* n. ssp. and 1140 for *L. baicalensis*). Phylogenetic trees were constructed under different optimality criteria (neighbour joining (NJ), minimal evolution (ME) and maximum parsimony (MP)) as integrated in MEGA 3.1 (Kumar et al., 2004). TREE-PUZZLE 5.2 (Schmidt et al., 2002) and MrBayes 3.1 (Huelsenbeck and Ronquist, 2001) were employed for maximum likelihood (ML) and Bayesian analyses, respectively. Sequences of the marine species *Pseudaxinella reticulata*, *Microciona prolifera* and *Scopalina ruetzleri* were included as outgroups. Bootstrap analysis with 1000 repetitions was performed under ML, MP and ME approaches in MEGA 3.1; 10,000 quartet puzzling steps were performed in TREE-PUZZLE 5.2 and at least 1,000,000 generations were generated in MrBayes 3.1.

3. Results

3.1. Selection of sponges

In the current study, four sponges from Lake Poso and Lake Matano included two morphologically different sponges belonging to the genus *Pachydictyum*. One was the previously described *Pachydictyum globosum* Weltner, 1901 and the other was a similar but as yet undescribed *Pachydictyum globosum* n. ssp. (later to be described as a proper species). These two *Pachydictyum* were morphologically very close but not identical (Fig. 1c–e). The other two sponges, originating from Lake Matano, were identified as *Nudospongilla vasta*. These two sponges differed in shape and global morphology from each other but were uniform in spicule shape and megascleres architecture. Their megascleres were similar to those from *Nudospongilla vasta* (Weltner, 1901), but no gemmules could be found, thus the species definition meets the same problem as with the Baikal sponges, were gemmules are also absent. Furthermore, five sponge specimens from Lake Baikal and one from Lake Dzhegatai Kül (*Baikalospongia dzhegatajensis*) were included. The sponges from Lake Baikal were identified as *Baikalospongia bacillifera* Dybowski, 1880, *Baikalospongia intermedia* Dybowski, 1880, *Lubomirskia abietina* Schwartschewski, 1901, *Lubomirskia baicalensis* Pallas 1771 and *Swartschewskaia papyracea* Dybowski, 1880. Identification of all species was first based on a thorough morphological characterization (Fig. 1f–l). The Spongillidae have a worldwide distribution, whereas the families Lubomirskiidae and Malawispongiidae are endemic or at least are geographically restricted. Therefore, we included several additional samples, *Ochridospongia* sp., and a number of different individuals of common Spongillidae. The latter were identified as *Ephydatia muelleri*, *E. fluviatilis*, *Eunapius fragilis* and *Eunapius carteri*, as *Spongilla lacustris* and *Trochospongilla horrida*. For details and references see Table 1.
different Pachydictyum globosum. Surprisingly, COI sequences recovered were not consistent with the morphological assignment of different species and genera. For example, Ephydatia muelleri, Baikalospongilla intermedia and Lubomirskaibaicaensis shared identical sequences with each other as did E. cooperensis (from N America) with E. fluviatilis 1 and 2 from Germany and with B. dzegeyatensis and Swartschewskia papyrus, moreover the Malawispongidae Ochridaspongia sp. was part of this group. The metaniid sponge Corvomeyenia sp. was at a basal position within the spongillids. The Sulawesian sponges, Pachydictyum globosum, Pachydictyum globosum n. ssp. and Nudospongilla vasta 1 and 2, were joined in a monophyletic group together with Trochospongilla species. This clade was the second-most deeply branching cluster among freshwater sponges. The position of an Ephydatia sp. which we collected from a German site remained unresolved in clade E since it differed at two distinct positions both from the E. fluviatilis and the E. muelleri sequences. While the basic topology of all reconstructed trees was identical, we found that the separation power of the COI data was not very high and some branches did not receive high statistical support due to the similarity among the compared sequences.

Deducing the encoded amino acids from COI sequences, the presence of translational exceptions was apparent, since UGA frequently codes for tryptophan. There are four tryptophan codons, at positions 114–116, 180–182, 249–251 and 429–431 within the partial 486 bp COI fragment. However, we noticed differences in the frequency of how often UGA is actually used as tryptophan codon. In the marine sponges Pseudaxinella reticulata and Scopalina ruezleri as well as Corvomeyenia sp. UGA was present in all four cases. For the freshwater sponges (except Corvomeyenia sp.) the triplet UGA was present three times, at the first, second and forth position, and UGG present once (249–251). In Microciona prolifera, UGG was present for the first occurrence (114–116), different from all other species studied here.

3.3. Molecular phylogenies based on 18S rDNA

Phylogenies employing the 18S rDNA were similar to but not identical to those obtained using COI data: Corvomeyenia sp. was again the most deeply branching species among freshwater sponges and the clade consisting of Pachydictyum globosum, Nudospongilla vasta, Trochospongilla horrida and Trochospongilla pennsylvanica was located at the second-most basal position within the Spongillidae (not shown). The two individual Spongilla lacustris 2 and 3 from Germany, the two Ephydatia muelleri, one from North America (E. muelleri 1) and one from Germany (E. muelleri 2), as well as the two individual Nudospongilla vasta 1 and 2, each shared an identical 18S rDNA with each other, as we had found for their COI sequences. Another set of species with identical sequences consisted of Baikalospongia intermedia, B. bacillifera and Lubomirska abaetina. These Lubomirskiidae (including Swartschewskia) formed a clade with Ephydatia muelleri as its sister-taxon, which is different from the COI analysis, however support at this level in the tree was poor. The Swartschewskia papyrus 18S rDNA varied at three positions from the E. fluviatilis or E. cooperensis sequence.

3.4. Inference of molecular phylogenies based on concatenated sequences

Inference of phylogenetic relationships between freshwater sponges based on either the 18S rDNA or COI data alone suffered from the high similarity among all compared sequences. For instance, in the 18S dataset, the two least related freshwater species, Lubomirska abaetina and Corvomeyenia sp. had still 1654 of 1683 bp in common (98% identity, 29 divergent sites), whereas the differentiation between Pachydictyum globosum and Nudospongilla vasta was based on only two divergent positions. Since the results obtained on basis of COI and 18S were very similar, the two datasets were concatenated for those 21 specimens for which both sequences were available, resulting in a 2177 bp alignment. In this comparison, Lubomirskaabaetina and Corvomeyenia sp. still had an identity of 97.8%, however, this is statistically more robust since it is based on 2122 shared and 47 divergent, i.e. phylogenetically informative sites.

Based on this alignment, very robust results were obtained in the ML analysis with Tree-Puzzle as indicated by the support values of >96 for all nodes except C and D (Fig. 3). Identical tree topologies were obtained under all models employed except MP and nodes C and D which were reversed in the Bayesian compared to the non-Bayesian analysis (Fig. 3). The statistical support was somewhat lower for MP, caused by the smaller number of parsimony-informative sites. All freshwater sponges except Corvomeyenia belonged to one of three larger clades labeled (i)–(iii), each beginning with the node E, H or J in Fig. 3 and each containing different Spongillidae. These groups or clades are (i) Malawispongidae (except Ochridaspongia sp. which was not present in the concatenated analyses), two different Trochospongilla and Nudospongilla; (ii) a joint clade consisting of the two genera Eunapius and Spongilla, each represented by two sequences; (iii) all Lubomirskiidae and all species belonging to the genus Ephydatia.

4. Discussion

From a molecular point of view, sponges are strongly undersampled, resulting in a lack of molecular phylogenetic markers for sponges and of sequence information in general. The exact position of several sponge taxa and the monophyly of the phylum Porifera is a matter of debate (Adams et al., 1999; Borchierielli et al., 2004,2001; Cavalier-Smith et al., 1996; Collins, 1998; Medina et al., 2001). To date, about 7500 sponge species have been described and the same number is expected still to await discovery (Hoo-
To speed up the identification process of both known and new species, a combination of morphological and molecular methods would be desirable. Mitochondrial COI gene 5' end sequences were suggested as molecular marker for barcoding eukaryotic organisms (Blaxter, 2004; Hebert et al., 2003). The COI gene is ubiquitous; in several groups of organisms such as birds, polychaetes or insects, it was shown to be well conserved but also to be variable enough to serve as phylogenetic and taxonomic marker. Mitochondrial markers have been introduced recently for sponge systematics (Addis and Peterson, 2005; Nichols, 2005; Schroeder et al., 2003). Based on the here presented data set, we extend these studies by the observation of translational exceptions within the sponge mitochondrial COI gene, in which the opal codon UGA frequently encodes tryptophan instead of terminating translation. While this finding alone is in consent with the mitochondrial codon usage of most invertebrates, including the closely related cnidarians, it is interesting to note that the extent and frequency of these exceptions correlates with the results of the phylogenetic analysis. For instance, Corvomeyenia sp. is at the most basal position among freshwater sponges (Fig. 2) and it has all four translational exceptions in common with the marine sponges *P. reticulata* and *S. ruetzleri*, whereas all other freshwater sponges use the triplet UGA three times but once UGG to code for tryptophan. However, more work on longer mitochondrial sequences is required to see to what extent these translational exceptions correlate with phylogeny.

The critical test of DNA barcoding is whether it enables discrimination between closely related species. Comparison of 5' COI sequences from 13,000 pairs of congeneric species showed a mean divergence of 11.3% (Hebert et al., 2003). With the striking exception of some cnidarians (sea anemones, corals, and some jellyfish), 98% of the species pairs exhibited sequence divergences greater than 2%. Since
sponges are a sister phylum to cnidarians it may not surprised that we found also for the here investigated different species of freshwater sponges a COI sequence divergence of only 2% as the maximum. While additional porifera COI sequences could be expected helpful for barcoding, sponge COI here did not allow discrimination between closely related species. COI sequences appeared to be too conserved also in an analysis of populations of the marine demosponges Crambe crambe in the Mediterranean and eastern Atlantic (Duran et al., 2003) and of Astrosclera willeyana in the Red Sea and Indo-Pacific (Wörheide, 2006). Thus, COI alone appears not as a particularly suitable marker for barcoding closely related sponges from the sea or freshwater. As an alternative, it should be used in combination with other molecular markers. In fact, the degree of sequence divergence observed here was about the same in case of COI and of 18S rDNA, encouraging us to concatenate the two datasets. With a similar approach, robust phylogenetic trees were previously obtained for 5 species of Demospongiae (Addis and Peterson, 2005).

Our data extend the existing information on the phylogeny and taxonomy of freshwater sponges. For instance, based on the analyses presented in Figs. 2 and 3, there is no clade supporting Spongillidae as a monophyletic group. The metaniid sponge Corvomeyenia sp. was previously suggested basal to the spongillids (Addis and Peterson, 2005). That hypothesis depended upon the method of analysis and sequence and was found in two of three analyses of COI and of 18S rDNA + COI analysis, whereas two of three analyses of 18S rDNA weakly supported Trochospongilla pennsylvanica as the basal taxon. Our study confirmed the hypothesis by Addis and Peterson (2005), since all analyses provided strong support for Corvomeyenia sp. as the most basal spongillid species, on basis of the concatenated data set as well as of 18S rDNA and COI alone.

Another set of interesting observations was made for the genus Ephydatia. COI sequences were identical for E. fluviatilis 1 and 2 from two different sites in Northern Germany with the published sequence of E. cooperensis. In contrast, the 18S rDNA sequence of E. fluviatilis 3 (Richelle-Maurer et al., 2006) differed in one nucleotide very close to the 3’ end from that of E. cooperensis (Peterson and Addis, 2000). This single nucleotide difference has previously been used as evidence to differentiate these two species. When we compared E. fluviatilis 3 with E. fluviatilis 1 and 2 and E. cooperensis, an identical sequence was found also for the 18S rDNA in the here compared region. Therefore, we analyzed 12 additional samples of E. fluviatilis for the critical 18S rDNA 3’ end (not shown), which were all found to be identical with E. cooperensis, E. fluviatilis 1 and 2 and to the exclusion of the E. fluviatilis 3 sequence. Thus, in this case, their very high genetic similarity as indicated by the shorter COI fragments was confirmed. Although a single nucleotide difference might result from errors, especially at the very beginning or very end of a sequence, this result might indicate the presence of microheterogeneity within the E. fluviatilis 18S rDNA 3’ end. Whether species with identical or one base maximum difference in the 18S rDNA and COI sequences are separate species cannot be decided. Previous analysis of the ITS2 rDNA region showed a difference of 10 nt between E. fluviatilis and E. cooperensis as well as between B. bacillifera and L. baicalensis (Addis and Peterson, 2005). Thus, the more variable ITS rDNA region appears more suitable as marker at this level. We collected one Ephydatia sp. from a German site which differed at two distinct positions from the E. fluviatilis, E. cooperensis and the E. muelleri COI sequences, and hence was placed at an intermediate position (see Fig. 2). Based on its spicule morphology, we were not able to assign it to either of the known species. Hence this specimen might represent a distinct species or result from hybridization and recombination between E. fluviatilis and E. muelleri. Further work including additional loci such as ITS rDNA would be required to resolve its position but was beyond the scope of the current study.

Our analyses included four of the six currently recognized families of living freshwater sponges (Manconi and Pronzato, 2002) and provide some clues for the possible evolution and phylogenetic affiliation of endemic species. The Malawispongidae family (Manconi and Pronzato, 2002) is based on weak morphological characters, such as the absence of gemmules and contains five monotypic genera, Malawispongia, Cortispongilla, Ochridaspongia, Pachydictyum and Spinospigongilla, all inhabiting ancient tectonic lakes in Africa, Europe and Asia (Malawi, Tanganyika, Kinneret, Ohrid and Poso). The wide geographical distance of these isolated regions makes a close phylogenetic relationship between these genera doubtful. Indeed, our analysis of COI from Ochridaspongia sp. surprisingly suggested this sponge as associated to the Ephydatia-Lubomirskiidae radiation rather than being closely related to Pachydictyum, making the Malawispongidae polyphyletic. Moreover, the DNA data do not support a close relatedness of the Sulawesi sponges Pachydictyum and Nudospongilla to Spongilla but rather to Trochospongilla since we found in all analyses high support for a joint clade of Pachydictyum, Nudospongilla and Trochospongilla. Within this clade, the genus Trochospongilla is paraphyletic with the genera Pachydictyum and Nudospongilla more closely related to T. pennsylvanica than to T. horrida. Our data suggests a common ancestor to group (i) in Fig. 3.

The origin of sponges from Lake Baikal has been enigmatic until recently. Spicules of the endemic Lubomirskiidae have been found in Late Miocene sediments (at least 24 MY old). Detailed records of sponge spicules in sediments from drilling cores of Lake Baikal (Martinson, 1936, 1938, 1940, 1948) demonstrate that it was possible to distinguish the Lubomirskiidae taxa at a species level (although Martinsons determination of four Spongillidae species on account of isolated spicules should be considered with caution). Thus, according to the fossil record, the Lubomirskiidae ought to be an old still extant taxon of
endemic freshwater sponges. Previously, the degree of nucleotide substitutions within the 18S rDNA of different Baikalian sponges was considered too low to draw unequivocal conclusions (Itskovich et al., 1999). However, with ~630 nt the 18S rDNA sequences had been very short in that analysis. In our COI-18S rDNA concatenated dataset, Lubomirskiiidae had between 2161 (Baikalospongia bacillifera) and 2167 (Swartschewskia papyracea) of the compared 2169 nt in common with Ephydatia fluviatilis, corresponding to a 99.6–99.9% sequence identity. In contrast, Spongilla lacustris had a 1.2% sequence divergence from the Lubomirskiiidae (2145–2147 shared positions), and was separated into a different clade (ii) in Fig. 3. Therefore, our data show that the sponges from Lake Baikal branch off from the other Spongillina together with Ephydatia fluviatilis, E. cooperensis and E. muelleri. This is essentially what Addis and Peterson (2005) have found, thus confirming and extending those results. Thus, all available data strongly indicate a common origin of the endemic Lubomirskiiidae together with the ubiquitous genus Ephydatia whereas the genera Ephydatia and Baikalospongia are not monophyletic. Moreover, the close relatedness between Lubomirskiiidae and the genus Ephydatia constitutes an interesting parallel to the Malawispongiidae in which Pachydictyum is more closely related to the ubiquitous genus Trochospongilla. Within the genus Ephydatia, coherence exists in the E. cooperensis–E. fluviatilis part as indicated by the identity between E. cooperensis and the two E. fluviatilis of Central European origin, even in the concatenated sequences. Thus, E. cooperensis, although it lacks the ability of gemmule formation, might be a more recent split off from the cosmopolitan E. fluviatilis.

Indirectly, our data has shed some light on the origin and loss of gemmules. Our data reinforce a view that the loss of gemmules may well have happened several times during the evolution of endemic taxa from the Spongillidae. The cosmopolitan Spongillidae have been found as isolated spicules in freshwater sediments of the Mesozoic and possibly also from Late Palaeozoic, whereas the oldest fossil gemmules date at least 112 MY, to the Early Cretaceous (Volkmer-Ribeiro and Reitner, 1991). However, the ability to form gemmulae might be traced back to the origin of the Spongilla, going along with, or even as a prerequisite for, the sponge colonization of freshwater environments. For instance, there might be no evolutionary advantage for gemmules in a stable lake environment, such as Lake Baikal. Gemmules are absent in the Lubomirskiiidae, and even the Baikalian Spongillidae show a tendency to reduced gemmule formation, probably due to the stable environmental conditions of Lake Baikal. Finally, our analyses clearly support the monophyletic origin of freshwater-adapted sponges with the outgroups chosen and small number of species included. Endemic species of different lake systems seem to have originated from a cosmopolitan founder species such as Ephydatia and Trochospongilla more frequently and independently as previously thought.

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