Addition to Sweden’s freshwater sponge fauna and a phylogeographic study of *Spongilla lacustris* (Spongillida, Porifera) in southern Sweden

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Abstract. Freshwater sponges constitute an overlooked part of the freshwater fauna in Sweden and there has been no recent systematic survey. Hitherto three species have been found in Sweden: *Spongilla lacustris* (Linnaeus, 1759), *Ephydatia fluviatilis* (Linnaeus, 1759) and *E. muelleri* (Lieberkühn, 1856). Neighbouring countries (Norway, Denmark, Estonia) harbour at least one additional species. We present a study on freshwater sponge diversity and distribution in the southern half of Sweden. We hypothesized dispersal within catchments to be less constrained than between, even at shorter intercatchment than intracatchment distances, and, as result, genetic distances being greater between than within catchments. We collected and identified freshwater sponges from 34 sites, using morphological and molecular data (coxI, 28S rRNA gene). We can report the presence of *Eunapius fragilis* (Leidy, 1851) in Sweden for the first time, and that *S. lacustris* is the most abundant and widely distributed freshwater sponge in Sweden. Genetic markers were tested on *S. lacustris* individuals for a phylogeographic study. From the 47 primers (24 markers), one pair presented successful amplification and enough variation for phylogeographic studies – i56, an intron located in a conserved gene. Seven different variants were found in the sampling area, but no clear population structure was observed.

Keywords. Spongillida, phylogeography, bar coding, EPIC marker, freshwater.

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Introduction

The freshwater sponge fauna makes only a small fraction of the global diversity of Porifera Grant, 1836. Recent assessment gives 219 valid species of freshwater sponges (Manconi & Pronzato 2008), all in the exclusively freshwater clade Spongillida Manconi & Pronzato, 2002, compared to a total
of ca 9400 valid species in World Porifera Database (de Voogd et al. n.d.). Thus, freshwater sponges constitute ca 2.3% of the global valid-species richness. On a national scale, we note that this fraction is also approximately true for Sweden where hitherto three out of 150 species (2%) are freshwater species (SLU Artdatabanken n.d.).

Sweden is a land of lakes. There are ca 107 700 lakes larger than 10^4 m^2 registered in SVAR – Svenskt vattenarkiv (Westman et al. 2017) to which we can add maybe twice as many smaller lakes and tarns (Håkanson 1994). Combine this with ca 197 000 km of streams and rivers of various size and regimes (Eklund 2010), over a geographical area of 407 000 km^2 (SCB n.d.) and a latitudinal gradient from ca 55° N to 69° N, and there are ample opportunities for freshwater sponges. Despite being an important part of the freshwater ecosystem (Manconi & Pronzato 2008), the Swedish freshwater sponge fauna has been somewhat overlooked (similar to the situation in the UK; Evans & Montagnes 2019). It was most recently reviewed by Arndt (1932), who in a collection-based study found three species in the fauna: Spongilla lacustris (Linnaeus, 1759), Ephydatia fluviatilis (Linnaeus, 1759) and E. muelleri (Lieberkühn, 1856). He also speculated that perhaps Eunapius fragilis (Leidy, 1851) [as Spongilla fragilis] and maybe also Trochospongilla horrida Weltner, 1893 could be present in Sweden. Since Eunapius fragilis has been reported from Denmark (Tendal 1967a, 1967b), Norway (Ökland & Ökland 1996) and Estonia (Lopp et al. 2007), it is likely that it has been overlooked in Sweden. The first aim of the present study is thus to investigate the presence of Eunapius fragilis in Sweden.

In addition to environmental parameters to explain the distribution of a species (Ökland & Ökland 1996; Evans & Montagnes 2019) there is also the historical component, which Avise and co-workers (Avise et al. 1987) designated intraspecific phylogeography. There have not been many studies of phylogeography in marine sponges (Wörheide et al. 2002; Duran et al. 2004; Nichols & Barnes 2005; Becking et al. 2013; Pasnin et al. 2020) and but a few for freshwater sponges (Schröder et al. 2003; Lucentini et al. 2013). Although assessing phylogeography in the marine environment is an aim, the external factors affecting dispersal are rarely explicitly clear, making it difficult to erect prior hypotheses of expected patterns. Turning to freshwater sponges in Sweden, two features make them a palatable pilot study. Firstly, freshwater sponges possess gemmulae, which are important for the sponges’ successful colonisation of freshwater, which is more prone to, e.g., drought and freezing than seawater. Gemmulae are persistent resting bodies that are also passive dispersal units (Manconi & Pronzato 2007, 2016). Secondly, there is a pattern of mainly west-to-east catchment areas in Sweden that could predict a distribution pattern. As shown by Dröscher & Waringer (2007), water body connectivity is an important abiotic factor to explain variability in sponge distribution. A reasonable hypothesis to test for an organism with water-borne passive dispersal is that specimens within the same catchment area are more closely related with each other than with specimens in different catchment areas, even if the geographical distance is shorter.

An obstacle to phylogeographic studies in sponges is to find markers with suitable variation within the study area; as the substitution rate in the mitochondrial genome is very low compared to most other taxa, the otherwise preferred mitochondrial markers show too little variation in sponges. Furthermore, due to the high content of symbionts in sponges, using otherwise useful anonymous markers such as RAPD (Williams et al. 1990) or RADSeq (Davey et al. 2010) may thus be problematic. Short of the extensive task of developing microsatellite primers, a second aim of the present study is to examine markers previously listed as potentially useful for phylogeography in other taxa (Chenuil et al. 2010; Gérard et al. 2013) and apply those to the in Sweden widespread species Spongilla lacustris, and assess whether these markers indicate a closer relationship within than between catchment areas.
Material and methods

Collecting material

Freshwater sponge specimens were collected in June–October between 2013 and 2017 (with a single specimen added in 2021) in a pattern mainly aimed to retrieve specimens of *Spongilla lacustris* from several sites in different catchment areas, and at some substantial distances between the sites. The area with sampling sites is ca 500 km by 450 km (Fig. 1). Sites were selected from maps with no more than a 15 minute hike from an access road, and were visited only once. At each site, all substrates were sampled at an area corresponding to 50–100 m shoreline (depending on conditions) down to ca 1 m depth, using aquascopes and waders where conditions permitted, otherwise from the beach, during at least 30 minutes. All sponge specimens found at a site were collected. One exception from the above sampling is the site in the Baltic (Helmarsvikken), where SCUBA was used.

The specimens were preserved in 99% ethanol. Approximately 24 h after collection, the alcohol was replaced once to ensure that the concentration of the alcohol the specimens were stored in was high enough to preserve DNA.

A small amount of sponge tissue was dissolved in bleach (sodium hypochlorite, ~6 %) until the solution became homogeneous. The bleach was discarded, and the spicules washed with 1) distilled water, 2) 70% ethanol and 3) 96% ethanol, sequentially. A droplet of the ‘spiculae slurry’ was applied on a microscope slide and dried on a heating block at 50°C, embedded in Canada balsam (Sigma-Aldrich) covered with a cover slip.

Slides were observed under an Olympus BX50 microscope, and photographed with a Nikon digital sight DS-Vi1 camera using NIS-Elements F 3.0 software. Spicule measurements were done on three randomly selected specimens, for each spicule type (megascleres, microscleres and gemmuloscleres).

Specimens and slides were deposited as vouchers at the Museum of Evolution, Uppsala University (UPSZMC).

DNA extractions

DNA was extracted using two different methods. DNeasy Blood & Tissue Kit (Qiagen), according to the manufacturer’s protocol and a CTAB/chloroform protocol modified after Winnepenninckx (Winnepenninckx et al. 1993); specimens were ground using a pestle in 1 × TE buffer instead of using liquid nitrogen. DNA quality was inspected by electrophoresis on 1% agarose gels and concentration measured using NanoDrop and double stranded DNA broad range (Qubit 3.0). The DNA was diluted to obtain a working concentration around 10 μg/ml.

Amplification and sequencing

The mix for amplification in all cases included 1 × buffer (DreamTaq Buffer, Thermo Scientific), 0.3 mM deoxyribonucleotide triphosphate (dNTP Mix), 0.2 mg/ml bovine serum albumin (BSA), 0.4 μM of each primer, 0.05 U/μl of Taq Polymerase (DreamTaq, Thermo Scientific), and 2 μl of DNA in a final volume of 25 μl. For the barcoding, it also included 2 mM MgCl₂, whereas a varying concentration of MgCl₂ was tried when trying markers for phylogeography. PCR was done in an Applied Biosystem Veritii 96-Well Thermal Cycler and quality checked on a 1.5% agarose gel. ExoSAP-IT diluted 1:10 was used to purify PCR products, which were sent to Macrogen, the Netherlands, for double-ended sequencing using the same primers as in PCR. Contigs were assembled and proof-read using SeqMan Pro ver. 14.1.0 (Burland 1999). Assembled sequences were checked against GenBank collections using standard BLAST (Madden 2013).
Fig. 1. Sampling sites and species found in main catchment areas in Sweden. Black lines represent watersheds between catchment areas, from the Swedish Meteorological and Hydrological Institute SVAR database (Westman et al. 2017). Crosses are sites visited but where no sponge was found, dots are sites where sponges were collected. The neighbouring pie icon indicates the species present at that site: blue (top right) is *Spongilla lacustris* (Linnaeus, 1759), yellow (bottom right) *Eunapius fragilis* (Leidy, 1851), black (bottom left) *Ephydatia fluviatilis* (Linnaeus, 1759) and green (top left) *Ephydatia muelleri* (Lieberkühn, 1856).
Barcoding identification

For barcoding, the Folmer fragment of \textit{coxI}, as well as the D3–D5 region in the gene coding for 28S rRNA was used. PCR amplifications and sequencing were done with the primers LCO1490–HCO2198 (Folmer et al. 1994) for \textit{coxI} and RD3A–RD3r (McCormack & Kelly 2002) or Por28S-830F–Por28S-1520R for 28S (Morrow et al. 2011); primers are shown in Supp. file 1. The PCR cycling regime for \textit{coxI} Folmer fragment was 1 × 5 min @ 94°C, 5 × 1 min @ 94°C, 1 min 30 sec @ 45°C and 1 min 30 sec @ 72°C, 35 × (1 min @ 94°C, 1 min 30 sec @ 50°C, 1 min @ 72°C), and a final step of 5 min @ 72°C. For Por28S-830F/Por28S-1520R the regime was 1 × 5 min @ 94°C, 30 × (30 s @ 94°C, 30 s @ 53°C, 30 s @ 72°C) and a final step of 5 min @ 72°C, and for the RD3A/RD3r primer combination the regime was 30 × (30 s @ 95°C, 30 s @ 50°C, 2 min @ 72°C) and final step of 5 min at 72°C.

Marker search

To find usable marker(s) for the phylogeographic part, attempts to amplify several markers were made: mitochondrial ATP6 gene, \textit{ATPase} \(\beta\) intron, a \textit{coxI} fragment (I3-M11) downstream to the Folmer fragment (Swierts et al. 2017), and 21 different exon-primed-intron-crossing (EPIC) loci (Chenuil et al. 2010), as shown in Supp. file 1. The cycling regimes were 3 min @ 95°C, 36 × (30 s @ 94°C, 45 s @ 57°C, 90 s @ 70°C), 10 min @ 72°C for \textit{coxI} (I3-M11), 5 min @ 95°C, 35 × of (30 s @ 95°C, 45 s @ 42°C, 1.30 min @ 68°C), 10 min @ 72°C for ATP6 and 5 min @ 95°C, 35 × (30 s @ 95°C, 30 s @ 45°C, 45 s @ 72°C), 4 min @ 72°C for \textit{ATPase} \(\beta\). For the EPIC markers, a touch-down general regime was used that can be described as 2 min @ 94°C, 14 × (1 min @ 94°C, 1 min @ \(\theta\) \_anneal [decreased 1°C/cycle], 1 min @ 73°C), 25 × (40s @ 94°C, 40s @ \(\theta\) \_anneal, 1 min @ 72°C), 3 min @ 73°C. The annealing temperature, \(\theta\) \_anneal, was between 58°C and 68°C for the different primer pairs.

Sequence analysis

For barcoding identification, additional sequences of the four target species and sequences representing other possible species were downloaded from GenBank using a BLAST search. Additionally, sequences of \textit{Trochospongilla} were downloaded to be used as outgroups (Addis & Peterson 2005). Sequences were aligned using MUSCLE ver. 3.8.425 (Edgar 2004) and the alignment was visualised in AliView ver. 1.15 (Larsson 2014).

PAUP* ver. 4.0 (Swofford 2003) was used for phylogenetic analyses of \textit{coxI} and 28S (neighbor-joining trees based on HKY85 distances; Hasegawa et al. 1985), and to compute genetic distances (HKY85) and raw differences between i56 variants.

To visualize evolutionary relationships between individuals and variants in the phylogeographic study, phylogenetic networks were done using SplitsTree4 ver. 4.14.6 (Huson & Bryant 2006) and PopART ver. 1.7 (Leigh & Bryant 2015).

To evaluate the amount of population structure, an analysis of molecular variance (AMOVA) was done using Arlequin ver. 3.5.2.2 (Excoffier et al. 2005), assuming \textit{S. lacustris} specimens collected in the same catchment area were from the same population.

Distribution

Collection sites of specimens were plotted on a map of Sweden using QGIS ver. 3.10.12 (QGIS Development Team 2016) (Fig. 1).

To quantify the association of the sponge species found, we computed Sørensen-Dice indices (Dice 1945; Sørensen 1948) as
$S_{x,y} = \frac{2a}{2a + b + c}$

where $a$ is the number of sampling sites where both species $x$ and $y$ were found, $b$ is the number of sites where only $x$ was found and $c$ is the number of sites where only $y$ was found.

**Results**

**Taxonomic account**

Sponges were found and collected at 34 of 68 sites visited (Fig. 1), located in 12 main catchment areas. All specimens appeared healthy, without signs of necrosis. A total of 142 specimens were collected: 109 specimens of *Spongilla lacustris* (Linnaeus, 1759), 18 of *Ephydatia muelleri* (Lieberkuhn, 1856) and four of *E. fluviatilis* (Linnaeus, 1759). Finally, eleven specimens identified as *Eunapius fragilis* (Leidy, 1851) were found, which are new records for the freshwater sponge fauna in Sweden. Collected specimens are listed in Table 1. Some species were found co-existing at the same site (Table 2). Gemmulae were visible in specimens collected at the end of the sampling season (October), but not observed in sponges collected earlier (May–September).

**Phylum Porifera Grant, 1836**

**Class Demospongiae Sollas, 1885**

**Subclass Heteroscleromorpha Cárdenas, Pérez & Boury-Esnault, 2012**

**Order Spongillida Manconi & Pronzato, 2002**

**Family Spongillidae Gray, 1867**

**Genus Spongilla Lamarck, 1816**

*Spongilla lacustris* (Linnaeus, 1759)

![Fig. 2](image)

*Spongia lacustris* Linnaeus, 1759: 1348.

**Material examined** (109 specimens, Table 1)

**SWEDEN – Dalsland** • 3 specs; Lake Vänern, Mellerud, Sunannå harbour; 58.7092° N, 12.5072° E; 11 Oct. 2017; Chloé Robert and Raquel Pereira leg.; UPSZMC 188195, 188197, 188199 • 4 specs; Åklång, Dalslands kanal, Häverud; 58.8214° N, 12.4061° E; 11 Oct. 2017; Chloé Robert and Raquel Pereira leg.; UPSZMC 188201, 188203, 188205, 188207. – **Bohuslän** • 1 spec.; Bohuslän, Lake S Bullaresjön, Naverstad, Sundshult; 58.7403° N, 11.5743° E; 8 Oct. 2017; Chloé Robert leg.; UPSZMC 188185 • 3 specs; Lake S Bullaresjön, Östad; 58.7719° N, 11.5693° E; 8 Oct. 2017; Chloé Robert and Raquel Pereira leg.; UPSZMC 188189, 188191, 188193. – **Södermanland** • 5 specs; Lake Likstammen, Öster Malma; 58.9468° N, 17.1708° E; 29 Jun. 2013; Mikael Thollesson leg.; UPSZMC 188012, 188015 to 188018 • 9 specs; Lake Naten, Stjärnhof bath; 59.0774° N, 17.0129° E; 30 Jun. 2013; Mikael Thollesson leg.; UPSZMC 188023, 188029 to 188036 • 5 specs; Lake Kyrksjön, bath; 59.0923° N, 16.9951° E; 30 Jun. 2013; Mikael Thollesson leg.; UPSZMC 188024 to 188028 • 5 specs; Klämmingen, Solbacken; 59.1307° N, 17.2454° E; 29 Jun. 2013; Mikael Thollesson leg.; UPSZMC 188014, 188019 to 188022. – **Uppland** • 1 spec.; Krägga herrgård; 59.603° N, 17.394° E; 2 Aug. 2017; Chloé Robert and Raquel Pereira leg.; UPSZMC 188114 • 2 specs; Viks bath; 59.7346° N, 17.4639° E; 2 Aug. 2017; Chloé Robert and Raquel Pereira leg.; UPSZMC 188114; 188118. – **Västmanland** • 3 specs; Hammarskogs bath; 59.7636° N, 17.5762° E; 2 Aug. 2017; Chloé Robert and Raquel Pereira leg.; UPSZMC 188112 to 188124 • 4 specs; Flottsund; 59.7875° N, 17.6626° E; 2 Aug. 2017; Chloé Robert and Raquel Pereira leg.; UPSZMC 188107, 188115, 188120, 188121 • 1 spec.; Lake Erken, Norr Malma, Erken freshwater laboratory; 59.8353° N,
Table 1 (continued). Material examined in the present study. Abbreviations of Swedish provinces: Bhl = Bohuslän; Dlr = Dalarna; Dls = Dalsland; Hls = Hälsingland; Hrj = Härjedalen; Jmt = Jämtland; Mpd = Medelpad; Sdm = Södermanland; Sth = Stockholm; Upp = Uppland.

| Species          | Specimen ID   | Designation            | Coordinates       | Catchment area | GenBank accession numbers | Voucher number(s) |
|------------------|---------------|------------------------|-------------------|----------------|--------------------------|-------------------|
| *Spongilla       | P059-170830-1 | Storsjön, Galhammar, Jmt | 62°46.7' N        | Indalsälven    | coxl 28S i56 i56         | UPSZMC 188161,    |
|                  |               |                        | 14°25.93' E       |                |                          | 188162            |
|                  | P059-170830-9 |                        |                   |                |                          | UPSZMC 188183,    |
|                  |               |                        |                   |                |                          | 188184            |
|                  | P059-190702-1 | Storsjön, Slandrom, Jmt | 63°8.08' N        |                |                          | UPSZMC 188209     |
|                  |               |                        | 14°38.87' E       |                |                          |                   |
|                  | P059-190702-2 |                        |                   |                |                          | UPSZMC 188210     |
|                  | P059-190702-3 |                        |                   |                |                          | UPSZMC 188211     |
|                  | P059-190702-4 |                        |                   |                |                          | UPSZMC 188212     |
|                  | P059-170829-1 | Gissjön, Mpd           | 62°31.4' N        | Ljungan        |                          | UPSZMC 188147     |
|                  |               |                        | 16°10.74' E       |                |                          |                   |
|                  | P059-170829-2 |                        |                   |                |                          | UPSZMC 188148     |
|                  | P059-170829-6 | Sundsjön, Jmt          | 62°57.08' N       |                |                          | UPSZMC 188155,    |
|                  |               |                        | 15°9.74' E        |                |                          | 188156            |
|                  | P059-170829-7 |                        |                   |                |                          | UPSZMC 188157,    |
|                  | P059-170829-8 |                        |                   |                |                          | 188158            |
|                  | P059-130831-1 | Ångratörn, by Vikstenstorpet, His | 61°54.95' N | Ljusnan       |                          | UPSZMC 188159,    |
|                  |               |                        | 15°23.23' E       |                |                          | 188160            |
|                  | P059-130831-2 |                        |                   |                |                          | UPSZMC 188092,    |
|                  |               |                        |                   |                |                          | 188093            |
|                  | P059-130831-3 |                        |                   |                |                          | UPSZMC 188094,    |
|                  |               |                        |                   |                |                          | 188095            |
|                  | P059-170830-11| Hedevik, Hrj            | 62°24.5' N        |                |                          | UPSZMC 188096,    |
|                  |               |                        | 13°40.18' E       |                |                          | 188097            |
|                  | P059-170830-12|                        |                   |                |                          | UPSZMC 188165     |
|                  | P059-170830-14|                        |                   |                |                          |                   |
|                  | P059-170830-15|                        |                   |                |                          |                   |
|                  | P059-170830-16|                        |                   |                |                          |                   |
|                  | P059-170830-17|                        |                   |                |                          |                   |
|                  | P059-170830-18|                        |                   |                |                          |                   |
Table 1 (continued on next six pages). Material examined in the present study.

| Species            | Specimen ID | Collection site                    | GenBank accession numbers |
|--------------------|-------------|------------------------------------|---------------------------|
|                    |             | Designation            | Coordinates | Cox I | 28S | 156 | 156 variety | Voucher number(s) |
| Spongilla lacustris| P059-170830-19 | Dalälven, Grädö, Dlr | 60°14.83’ N 16°1.62’ E | Dalälven | OM105903 | y-290G | UPSZMC 188175 |
|                    | P059-170830-20 |                     |             |       |     |     |          |             |
|                    | P059-170830-21 |                     |             |       |     |     |          |             |
|                    | P059-170830-22 |                     |             |       |     |     |          |             |
|                    | P059-170830-23 |                     |             |       |     |     |          |             |
|                    | P059-130828-1 | Strands boat club, Dlr | 60°29.05’ N 15°44.68’ E |         |       |     |          |             |
|                    | P059-130828-2 |                     |             |       |     |     |          |             |
|                    | P059-130828-3 |                     |             |       |     |     |          |             |
|                    | P059-130828-4 |                     |             |       |     |     |          |             |
|                    | P059-130828-6 | STRAND BOAT CLUB, DLR | 60°30.78’ N 14°48.53’ E |         |       |     |          |             |
|                    | P059-130828-17 |                     |             |       |     |     |          |             |
|                    | P059-130828-19 |                     |             |       |     |     |          |             |
|                    | P059-130828-20 |                     |             |       |     |     |          |             |
|                    | P059-130828-10 | S Mojesjön, bathing place, Dlr | 60°36.02’ N 15°5.31’ E |         |       |     |          |             |
|                    | P059-130828-11 |                     |             |       |     |     |          |             |
|                    | P059-130828-12 |                     |             |       |     |     |          |             |
|                    | P059-130828-13 |                     |             |       |     |     |          |             |
|                    | P059-130828-14 |                     |             |       |     |     |          |             |
|                    | P059-130828-15 |                     |             |       |     |     |          |             |
Table 1 (continued). Material examined in the present study.

| Species                      | Specimen ID | Collection site                              | GenBank accession numbers |
|------------------------------|-------------|----------------------------------------------|---------------------------|
| *Spongilla lacustris*        | P059-130829-1 | Malungs camping, mouth of the river in Västerdalälven Dlr | UPSZMC 188072, 188073, UPSZMC 188074 |
|                              | P059-130829-2 | Orsåsjön, at the beach restaurant, Dlr | UPSZMC 188082 |
|                              | P059-130830-1 | Skattungen, Oresjöns boat club, Upp | UPSZMC 188083 |
|                              | P059-130830-2 | Hällsjön, northern resting place, Dlr | UPSZMC 188090 |
|                              | P059-130830-5 | Gimo dam, Upp | UPSZMC 188003 |
|                              | P059-170803-6 | Dock at Erken-lab, Upp | UPSZMC 188137, 188138, UPSZMC 188140, 188141 |
|                              | P059-170803-7 | Krägga mansion, Upp | UPSZMC 188213, 188214, UPSZMC 188114 |
|                              | P059-170802-11 | Viks badplats, Upp | UPSZMC 188110, 188111 |
|                              | P059-170802-12 | Hammarskogs badplats, Upp | UPSZMC 188112, 188113, UPSZMC 188122 |
|                              | P059-170802-8 | OL985663 | UPSZMC 188123, 188124, 188125 |

Table 1 (continued). Material examined in the present study.
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| Species         | Specimen ID | Designation          | Collection site | GenBank accession numbers | Coordinates | Catchment area | GenBank accession numbers | Coordinates | Catchment area | GenBank accession numbers | Coordinates | Catchment area | GenBank accession numbers | Coordinates | Catchment area | GenBank accession numbers | Coordinates | Catchment area | GenBank accession numbers | Coordinates | Catchment area | GenBank accession numbers | Coordinates | Catchment area | GenBank accession numbers |
|-----------------|-------------|----------------------|-----------------|---------------------------|-------------|----------------|--------------------------|-------------|----------------|--------------------------|-------------|----------------|--------------------------|-------------|----------------|--------------------------|-------------|----------------|--------------------------|-------------|----------------|--------------------------|-------------|----------------|--------------------------|-------------|----------------|--------------------------|
| *Spongilla*     | P059-170802-1 | Flottsund, Upp       | 59°47.25' N     | OL979179                  | 17°39.76' E | OL979179       | OM105923                  | y-290G      | UPSZMC 188107    |                          |              |                |                          |              |                |                          |              |                |                          |              |                |                          |              |                |                          |              |                |                          |
|                 | P059-170802-2 |                     |                 |                           |             |                |                          |             |                |                          |              |                |                          |              |                |                          |             |                |                          |              |                |                          |             |                |                          |             |                |                          |              |                |                          |
|                 | P059-170802-5 |                     |                 |                           |             |                |                          |             |                |                          |              |                |                          |              |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |
|                 | P059-170802-6 |                     |                 |                           |             |                |                          |             |                |                          |              |                |                          |              |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |
|                 | P059-140526-1 | Siggefora Lake, Upp | 59°58.55' N     | UPSZMC 188100,           | 17°9.42' E  | UPSZMC 188100,  |                          |             |                |                          |              |                |                          |              |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |
|                 | P059-140526-2 |                     |                 |                           |             |                |                          |             |                |                          |              |                |                          |              |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |
|                 | P059-140526-3 |                     |                 |                           |             |                |                          |             |                |                          |              |                |                          |              |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |
|                 | P059-140526-4 |                     |                 |                           |             |                |                          |             |                |                          |              |                |                          |              |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |
|                 | P059-170803-1 | Simbadet, Österbybruk, Upp | 60°11.7' N     | UPSZMC 188126,           | 17°54.86' E | UPSZMC 188126,  |                          |             |                |                          |              |                |                          |              |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |
|                 | P059-170803-2 |                     |                 |                           |             |                |                          |             |                |                          |              |                |                          |              |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |
|                 | P059-170803-3 |                     |                 |                           |             |                |                          |             |                |                          |              |                |                          |              |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |
|                 | P059-170803-4 |                     |                 |                           |             |                |                          |             |                |                          |              |                |                          |              |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |
|                 | P059-170803-5 |                     |                 |                           |             |                |                          |             |                |                          |              |                |                          |              |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |
|                 | P059-130629-1 | Likstammen, east Malma, Sdm | 58°56.81' N     | UPSZMC 188126,           | 17°10.25' E | UPSZMC 188126,  |                          |             |                |                          |              |                |                          |              |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |
|                 | P059-130629-2 |                     |                 |                           |             |                |                          |             |                |                          |              |                |                          |              |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |
|                 | P059-130629-3 |                     |                 |                           |             |                |                          |             |                |                          |              |                |                          |              |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |
|                 | P059-130629-4 |                     |                 |                           |             |                |                          |             |                |                          |              |                |                          |              |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |
|                 | P059-130629-5 |                     |                 |                           |             |                |                          |             |                |                          |              |                |                          |              |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |
|                 | P059-130630-1 | Naten, Stjärnhof bathing place, Sdm | 59°4.64' N     | UPSZMC 188126,           | 17°0.77' E  | UPSZMC 188126,  |                          |             |                |                          |              |                |                          |              |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |
|                 | P059-130630-2 |                     |                 |                           |             |                |                          |             |                |                          |              |                |                          |              |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |
|                 | P059-130630-3 |                     |                 |                           |             |                |                          |             |                |                          |              |                |                          |              |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |             |                |                          |

**Table 1** (continued). Material examined in the present study.
### Table 1 (continued). Material examined in the present study.

| Species       | Specimen ID | Collection site          | Designation | Coordinates | GenBank accession numbers | Voucher number(s) |
|---------------|-------------|--------------------------|-------------|-------------|--------------------------|-------------------|
| *Spongilla lacustris* | P059-130630-4 | | | | OM105900 z-242T | UPSZMC 188031 |
|               | P059-130630-5 | Kyrkšön, bathing place, Sdm | 59°5.54' N 16°59.71' E | OL979189 OL985652 | UPSZMC 188032 |
|               | P059-130630-6 | Kyrkšön, bathing place, Sdm | 59°5.54' N 16°59.71' E | OM105914 w-377G |
|               | P059-130630-7 | Klämminingen, Solbacken, Sdm | 59°7.84' N 17°14.72' E | OL979203 OL985664 OM105926 w-377G |
|               | P059-130629-6 | Åklång, Dalslands kanal, Håverud, Dls | 58°49.28' N 12°24.37' E | UPSZMC 188024 |
|               | P059-130629-7 | Åklång, Dalslands kanal, Håverud, Dls | 58°49.28' N 12°24.37' E | UPSZMC 188025 |
|               | P059-130629-8 | Åklång, Dalslands kanal, Håverud, Dls | 58°49.28' N 12°24.37' E | UPSZMC 188026 |
|               | P059-171011-1 | Vänern, Sunnannå Hamm, Mellerud, Dls | 58°42.55' N 12°30.43' E | UPSZMC 188027 |
|               | P059-171011-6 | Vänern, Sunnannå Hamm, Mellerud, Dls | 58°42.55' N 12°30.43' E | UPSZMC 188028 |
|               | P059-171011-7 | Åklång, Dalslands kanal, Håverud, Dls | 58°49.28' N 12°24.37' E | UPSZMC 188029 |
|               | P059-171011-8 | Åklång, Dalslands kanal, Håverud, Dls | 58°49.28' N 12°24.37' E | UPSZMC 188030 |
|               | P059-171011-9 | Åklång, Dalslands kanal, Håverud, Dls | 58°49.28' N 12°24.37' E | UPSZMC 188031 |
|               | P059-171011-10 | Åklång, Dalslands kanal, Håverud, Dls | 58°49.28' N 12°24.37' E | UPSZMC 188032 |
|               | | | | | | |

Note: The table continues with additional entries that are not shown in the excerpt provided.
Table 1 (continued). Material examined in the present study.

| Species              | Specimen ID | Collection site | GenBank accession numbers |
|----------------------|-------------|-----------------|---------------------------|
|                      |             | Designation     | Coordinates              | GenBank accession numbers | Is6 | Is6 variety | Voucher number(s) |
| *Spongilla lacustris*| P059-171011-9 | Bullaresjön, Sundshult, Naverstad, Bhl | 58°44.42’ N 11°34.46’ E | OM105929 y-290G | UPSZMC 188207, 188208 |
|                      | P059-171008-1 | Bullaresjön, Ostad, Bhl | 58°46.31’ N 11°34.16’ E |
|                      | P059-171008-3 | | |
|                      | P059-171008-4 | | |
|                      | P059-171008-5 | | |
| *Eunapius fragilis*  | P059-170830-10 | Storsjön, Galhammar, Jmt | 62°46.7’ N 14°25.93’ E | UPSZMC 188163, 188164 |
|                      | P059-170830-2 | | |
|                      | P059-170830-3 | | OL979181 |
|                      | P059-170830-4 | | OL979180 OL985661 |
|                      | P059-170830-5 | | OL979202 OL985653 |
|                      | P059-170830-6 | | OL979209 |
|                      | P059-170830-7 | | OL979185 |
|                      | P059-170830-8 | | OL979210 OL985651 |
|                      | P059-170802-10 | Hammarskogs badplats, Upp | 59°45.82’ N 17°34.57’ E | UPSZMC 188108, 188109 |
|                      | P059-170802-3 | Flottsund, Upp | 59°47.25’ N 17°39.76’ E |
|                      | P059-170802-4 | | OL979197 |
| *Ephydatia muelleri* | P059-170829-3 | Gissjön, Mpd | 62°31.4’ N 16°10.74’ E | UPSZMC 188149, 188150 |
|                      | P059-170829-4 | | |
|                      | P059-170829-5 | | OL979190 OL985668 |
| Species          | Specimen ID | Collection site                           | Designation                  | Coordinates          | Catchment area | GenBank accession numbers | Voucher number(s) |
|------------------|-------------|-------------------------------------------|------------------------------|----------------------|----------------|--------------------------|------------------|
| *Ephydatia*      | P059-170828-1 | Delbo, Södra Dellensjön, Hls            | 61°14.85′ N 16°31.24′ E     | Delängersån          |                | OL979186 OL985654        | UPSZMC 188143, 188144 |
| *muelleri*       | P059-170828-2 | Hedevik, Hrj                             | 61°14.85′ N 16°31.24′ E     | Ljusnan              |                | OL979194 OL985657        | UPSZMC 188145, 188146 |
| *fluviatilis*    | P059-170830-13 | Dalälven, Grådö, Dlr                   | 60°14.83′ N 16°1.62′ E      | Dalälven             |                | OL985662                 | UPSZMC 188168, 188169 |
|                  | P059-130828-18 | Flosjön, boat site, Dlr                 | 60°36.02′ N 15°5.31′ E      |                      |                | OL985647                 | UPSZMC 188066, 188067 |
|                  | P059-130828-8 | S Mojesjön, bathing place, Dlr          | 60°36.02′ N 15°5.31′ E      |                      |                | OL985671                 | UPSZMC 188068, 188069 |
|                  | P059-130828-9 |                            |                             |                      |                | OL985666                 | UPSZMC 188070     |
|                  | P059-130829-3 | Malungs camping, mouth of the river in Västerdalälven, Dlr | 60°41.08′ N 13°42.14′ E |                      |                | OL985665                 | UPSZMC 188076, 188077 |
|                  | P059-130829-4 |                            |                             |                      |                | UPSZMC 188078, UPSZMC 188079 |
|                  | P059-130829-5 |                            |                             |                      |                | UPSZMC 188080, UPSZMC 188081 |
|                  | UP-16-2-2     | Dock at Erken-lab, Upp                  | 59°50.12′ N 18°37.99′ E     | Norrtäljeån          |                | UPSZMC 188244, UPSZMC 188244 |
|                  | UP-16-1-2     | Badplatz Svanberga: Erken-S bathing place drainage pipe, Upp | 59°50.22′ N 18°39.35′ E |                      |                | UPSZMC 188242, UPSZMC 188243 |
|                  | P059-171011-3 | Vännern, Sunanà Hamm, Mellerud, Dls    | 58°42.55′ N 12°30.43′ E     | Göta Älv             |                | UPSZMC 188237, UPSZMC 188238 |
|                  | P059-171011-5 |                            |                             |                      |                | UPSZMC 188239, UPSZMC 188240 |
|                  | P059-170902-1 | Helgaviken, Sth                         | 59°17.34′ N 18°42.19′ E     | Östersjön (the Baltic)|               | OL979208 OL985643       | UPSZMC 188229, 188230 |
|                  | P059-170902-2 |                            |                             |                      |                | UPSZMC 188231, UPSZMC 188232 |
|                  | P059-170902-3 |                            |                             |                      |                | UPSZMC 188233, UPSZMC 188234 |
|                  | P059-211231-1 |                            |                             |                      |                | Pending                  |                  |
Fig. 2. *Spongilla lacustris* (Linnaeus, 1759). A–B. Habitus. A. Specimen growing under a pontoon (P059-171011-6). B. Specimen growing on anchor chain, with finger-like projections (P059-170802-48). C–D. Spiculae. C. Megasclere (P059-171008-4). D. Microsclere (P059-171008-1). E–F. Estimated spicula size distribution within and between specimens, P059-130831-1 (solid line), P059-170802-8 (dashed) and P059-171008-4 (dotted). Marks on the x-axis represent spiculae measured. Brackets correspond to ranges in literature (red = Tendal 1967b; blue = Penney & Racek 1968; black = Evans & Montagnes 2019). E. Megascleres. F. Microscleres.
Table 2. Co-occurrence of freshwater sponges at different sites in the present study. On the diagonal are the number of sites where the species was found, lower triangle gives the number of co-occurrences, and upper diagonal shows Sørensen-Dice indices of species association (ranging from 0 – never found together to 1 – always found together). At two sites the three species *Spongilla lacustris* (Linnaeus, 1759), *Ephydatia muelleri* (Lieberkühn, 1856) and *Eunapius fragilis* (Leidy, 1851) were found together; at no site did all four species co-occur.

|                | *Spongilla lacustris* | *Ephydatia fluviatilis* | *Ephydatia muelleri* | *Eunapius fragilis* |
|----------------|-----------------------|-------------------------|----------------------|---------------------|
| *Spongilla lacustris* | 30                    | 0                       | 0.40                 | 0.18                |
| *Ephydatia fluviatilis* | 0                    | 1                       | 0                    | 0                   |
| *Ephydatia muelleri* | 8                    | 0                       | 10                   | 0                   |
| *Eunapius fragilis* | 3                    | 0                       | 0                    | 3                   |

Description

**Habitus.** Specimens usually bright green (Fig. 2A), often with finger-like projections (Fig. 2B). Surface hispid due to protruding spicules.

**Spiculae.** Skeleton with two classes of oxeas: smooth megascleres (Fig. 2C) and spined microscleres (Fig. 2D). Megascleres fusiform, microscleres acerate (Boury-Esnault & Rützler 1997). Gemmuloscleres
were not observed. Megasclere length 265 μm (190–375 μm), width 11 μm (2.5–20 μm) (Fig. 2E). Microsclere length 76 μm (50–117.5 μm), width 4.8 μm (2.5–7.5 μm) (Fig. 2F).

**Distribution and habitat**
Widely distributed, entire sampling area; hard substrates in lakes and rivers.

**Remarks**
The finger-like projections distinguish this species from the other Swedish freshwater sponges, as does the presence of microscleres.

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**Fig. 3. Ephydatia fluviatilis** (Linnaeus, 1759). A. Habitus (P059-211231-1). B. Spiculae, m = megasclere (P059-170902-2), g = gemmulosclere (P059-170902-1). C. Estimated megasclere size distribution within and between specimens, P059-170902-1 (solid line), P059-170902-2 (dashed), P059-170902-3 (dotted). Marks on the x-axis represent spiculae measured. Brackets correspond to ranges in literature (red = Tendal 1967b; blue = Penney & Racek 1968; black = Evans & Montagnes 2019).
Genus *Ephydatia* Lamouroux, 1816

*Ephydatia fluviatilis* (Linnaeus, 1759)

Fig. 3

*Spongilla fluviatilis* Linnaeus, 1759: 1348.

**Material examined** (4 specimens, Table 1)

SWEDEN – **Stockholm** • 3 specs; Helgarsviken; 59.289° N, 18.7032° E; 2 Sep. 2017; Raquel Pereira leg.; UPSZMC 188229, 188231, 188233 • 1 spec.; Stavsnäs vinterhamn; 59.2889° N, 18.7062° E; 31 Dec. 2021; Raquel Pereira and Jesper Svedberg leg.; UPSZMC 189246

**Description**

**Habitus.** Colour from pale to brownish, encrusting (Fig. 3A).

**Spiculae.** Megascleres and gemmuloscleres. Megascleres thin, smooth and acute oxeas (Fig. 3B), 250 μm (60–360 μm) long (Fig. 3C), 7.5 μm (2–15 μm) wide. Very few gemmuloscleres found in the collected specimens; birotulated, often a spine on the shaft, 20 μm long and 25 μm wide.

**Distribution and habitats**

This species was only found in the Baltic, but was missing from the bona fide freshwater samples.

**Remarks**

Five specimens from inland waters tentatively identified as *E. fluviatilis* (based on megascleres only, lacking gemmulae) was reassigned to *Eunapius fragilis* based on bar-coding sequences and a re-scrutiny of spiculae, so there is a possibility that some previously reported specimens of this (in Sweden) rare species may be misidentifications. It is quite possible, however, that it is present at greater depth at some of the sites, and thus missed in our study – the specimens from the Baltic were all from greater depths. We note, though, that Arndt (1932) only listed specimens from seven sites, all but one site outside (south of) the present study area, so the lack of observations may reflect a rare occurrence.

**Ephydatia muelleri** (Lieberkühn, 1856)

Fig. 4

*Spongilla mulleri* Liberkühn, 1856: 510.

**Material examined** (18 specimens, Table 1)

SWEDEN – **Dalarna** • 2 specs; Lake Vänern, Mellerud, Sunannå harbour; 58.7092° N, 12.5072° E; 11 Oct. 2017; Chloé Robert and Raquel Pereira leg.; UPSZMC 188237, 188239. – **Uppland** • 1 spec.; Lake Erken, Norr Malma, Erken freshwater laboratory; 59.8353° N, 18.6331° E; 30 May 2016; Raquel Pereira leg.; UPSZMC 188244 • 1 spec.; Lake Erken, bath Svanberga; 59.837° N, 18.6559° E; 30 May 2016; Raquel Pereira leg.; UPSZMC 188242. – **Dalarna** • 1 spec.; Dalälven, Grädö; 60.2471° N, 16.027° E; 28 Aug. 2013; Mikael Thollesson leg.; UPSZMC 188062 • 1 spec.; Dala-Floda, Lake Flosjön, boating site; 60.513° N, 14.8089° E; 28 Aug. 2013; Mikael Thollesson leg.; UPSZMC 188052 • 3 specs; Lake S Mojesjön, bath; 60.6003° N, 15.0885° E; 28 Aug. 2013; Mikael Thollesson leg.; UPSZMC 188066, 188068, 18807. • 3 specs; Malungs camping, outlet into Västerdalälven; 60.6847° N, 13.7023° E; 29 Aug. 2013; Mikael Thollesson leg.; UPSZMC 188076, 188078, 188080. – **Hälsingland** • 2 specs; Delsbo, Lake S Dellsjöön; 61.8097° N, 16.5706° E; 28 Aug. 2017; Raquel Pereira leg.; UPSZMC 188143, 188145. – **Härjedalen** • 1 spec.; Hedevikten; 62.4083° N, 13.6697° E; 30 Aug. 2017; Chloé Robert and Raquel Pereira leg.; UPSZMC 188168. – **Medelpad** • 3 specs; Lake Gissjön;
62.5233° N, 16.179° E; 29 Aug. 2017; Chloé Robert and Raquel Pereira leg.; UPSZMC 188149, 188151, 188153.

**Description**

**HABITUS.** Collected specimens had a bright green colour. Ridges on the surface are directed towards the centre of the sponge (Fig. 4A).

**SPICULAE.** This species presents two categories of spicules: megascleres and gemmuloscleres. Megascleres are fusiform and spiny oxeas. Spine abundance can be significant or limited on oxeas within the same individual (Fig. 4B). Usually, apices contain less spines than median part. Gemmuloscleres are birotulated. Shaft is terminated by rotules incised into smaller rays (Fig. 4B). Quantity of rays varies between specimens. Megascleres 255 μm (190–295 μm) long and 11.5 μm (5–22.5 μm) wide. A total

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**Fig. 4.** *Ephydatia muelleri* (Lieberkühn, 1856). A. Habitus (P059-170829-4). B. Spiculae, m = megascleres (P059-170830-13), g = gemmuloscleres (P059-130828-18). C. Estimated megasclere size distribution within and between specimens, P059-130829-3 (solid line), P059-170830-13 (dashed) and UP-16-1-2 (dotted). Marks on the x-axis represent spiculae measured. Brackets correspond to ranges in literature (red = Tendal 1967b; blue = Penney & Racek 1968; black = Evans & Montagnes 2019).
of 30 gemmuloscleres were measured with an average length and width of 16 μm (length 12.5–20 μm, width 15–20 μm).

**Distribution and habitats**

Wide distribution, entire sampling area, 17 from lakes, 1 from a river.

**Genus Eunapius** Gray, 1867

*Eunapius fragilis* (Leidy, 1851)

**Fig. 5**

*Spongilla fragilis* Leidy, 1851: 278.

**Material examined** (11 specimens, Table 1)

SWEDEN – **Uppland** • 1 spec.; Hammarskogs bath; 59.7636° N, 17.5762° E; 2 Aug. 2017; Chloé Robert and Raquel Pereira leg.; UPSZMC 188108 • 2 specs; Flottsund; 59.7875° N, 17.6626° E; 2 Aug. 2017; Chloé Robert and Raquel Pereira leg.; UPSZMC 188116, 188118. – **Jämtland** • 8 specs; Lake Storsjön, Galhammar; 62.7784° N, 14.4321° E; 30 Aug. 2017; Chloé Robert and Raquel Pereira leg.; UPSZMC 188163, 188215, 188217, 188219, 188221, 188223, 188225, 188227.

**Description**

**HABITUS.** Specimens of this species have a pale green colour, slightly lighter than the other three species (Fig. 5A).

**SPICULAE.** Megascleres and gemmuloscleres. Megascleres are smooth and fusiform oxeas (Fig. 5B). Gemmuloscleres are spiny and rod-shaped (Fig. 5B). Measured megascleres 219.5 μm (110 to 300 μm) long (Fig. 5C) and 6.7 μm (2.5 to 10 μm) wide. Few gemmuloscleres were present, 49 in two specimens were measured. Their average length was 85.5 μm (60 to 110 μm) (Fig. 5D) and width 5.6 μm, (2.5 to 7.5 μm).

**Distribution and habitats**

Specimens collected in southeast and northern part of sampling area (Fig. 1); no specimens found in the western part; from lakes and in a river close to lake inlet.

**Bar-code analyses**

There was a high within-species similarity in the specimens of the present study, and they fall well within the variation encountered in published sequences. Particularly **coxI** sequences were useful (Fig. 6A), whereas the use of **28S** (Fig. 6B) was hampered by fewer available sequences in the databases and that the available sequences in many cases were short; of the ca 660 bp amplified by the used primers, there was only ca 350 bp of useful overlap with most GenBank sequences. Both markers provided useful data to identify the species.

**Marker search**

Primer pairs tested for amplification are listed in Supp. file 1, but most of them failed to amplify *S. lacustris* DNA extracts. Different annealing temperatures and MgCl₂ concentrations were used, with the latter having no effect on the amplification results. No product was detected for 21 of the 24 primer pairs tested. The remaining three primer pairs were successful; i56-F/i56-R, i56-Spla-F/i56-Spla-R and ATP6porF/ATP6porR (primer pairs 23, 25 and 5 in Supp. file 1). The marker i56 is an intron located in a conserved gene coding for glutamyl-prolyl-tRNA-synthetase (Chenuil et al. 2010). Using the primer
pair i56-F/i56-R at an annealing temperature of 64°C (following tests of optimal temperature) product was obtained for 43 samples, and 40 of these were successfully sequenced.

The primer pair ATP6porF/ATP6porR, targeting the mitochondrial gene ATP6, amplified a region ca 450 bp long. However, as expected, a low amount of variation was detected between specimens, with less than 0.7% nucleotide variation. Thus, for this study, only primer pair i56-F / i56-R was selected.

**Genetic diversity**

For the i56 marker we found seven distinct sequence variants in the sampled *S. lacustris* populations, which we designate y-24G, y-290G, y-290A, z-242T, z-242G, w-377G and w-377T (see Table 3). The most abundant variant was y-290G, observed for 18 individuals collected in six different catchment areas.

![Fig. 5. Eunapius fragilis (Leidy, 1851). A. Habitus (P059-170829-4). B. Spiculae, m = megasclere (P059-170830-13), g = gemmulosclere (P059-170902-1). C–D. Estimated spicula size distribution within and between specimens, P059-170802-10 (solid line), P059-170830-2 (dashed) and P059-170830-4 (dotted). Marks on the x-axis represent spiculae measured. C. Megascleres. Brackets correspond to ranges in literature (red = Tendal 1967b; blue = Penney & Racek 1968; black = Evans & Montagnes 2019). D. Gemmuloscleres.](image)
Variants \( w-377G \) and \( z-242T \) were also common; 11 and 7 specimens, respectively, shared these variants, sampled from five and three different places. Remaining variants (\( y-24G \), \( y-290A \), \( w-377T \), \( z-242G \)) were found only for one or two specimens. Two specimens from the Ljusnan catchment area shared variant \( y-290A \), one individual from Dalälven had variant \( z-242G \), one from Göta Älv had \( w-377T \), and a single individual from the Svärtaån catchment area was found with \( y-24G \). Genetic distance and raw differences between variants are shown in Table 4. Variants \( y-290A \) and \( w-377T \) are least similar, with 24 segregating sites and a 2.7% HKY85 nucleotide difference. Least difference distance is found between \( y-290G \) and \( y-290A \), \( z-242T \) and \( z-242G \), and \( w-377G \) and \( w-377T \) with just one segregating site.

**Phylogenetic network**

In a phylogenetic network we see three main clusters; \( y-24G \) and \( y-290G \) with \( y-290A \), \( w-377G \) with \( w-377T \), and \( z-242T \) with \( z-242G \) (Fig. 7).

As PopArt does not handle gapped sites, parts of the alignment containing gaps were thus ignored in the analysis. Thus, in the median joining network, variants \( z-242T \) and \( z-242G \) are seen as identical (Fig. 7A) as they differ only in indel sites. In the majority of catchment areas (Svärtaån, Norrström, Göta Älv)

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**Fig. 6.** Neighbor-joining trees based on HKY85 distances. **A.** \( \text{coxI} \) dataset. **B.** 28S dataset. Sequences with a specimen field number (starting with \( \text{P052-} \) or \( \text{P059-} \)) are from the present study, remaining sequences are from GenBank with the accession number in the label.
Table 3. Variable sites in the 460 bp long alignment of i56 sequences from *Spongilla lacustris* (Linnaeus, 1759). Seven varieties, designated z-242T, z-242G, y-290G, y-290A, y-24G, w-377G and w-377T, are found, with a total of 27 variable sites in the alignment. A dot indicates identity with the reference sequence (z-242T), a dash indicates an indel, and N is the number of specimens found of this variety.

|   | 10 | 24 | 52 | 57 | 60 | 61 | 62 | 63 | 64 | 65 | 66 | 67 | 71 | 91 | 128 | 134 | 155 | 182 | 197 | 239 | 240 | 241 | 242 | 271 | 281 | 290 | 377 | 392 | N |
|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| z-242T | C | A | C | A | T | C | C | A | T | G | C | T | G | T | C | G | G | A | T | G | – | A | G | C | 7 |
| z-242G | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | 1 |
| y-290G | T | . | T | – | – | – | – | – | – | – | – | – | T | A | A | . | A | A | . | . | . | . | A | G | . | A | 18 |
| y-290A | T | . | T | – | – | – | – | – | – | – | – | – | T | A | A | . | A | A | . | . | . | . | . | . | A | 2 |
| y-24G | T | G | T | – | – | – | – | – | – | – | – | – | T | A | A | . | A | A | . | . | . | . | . | . | . | A | 1 |
| w-377G | T | . | – | . | . | . | . | . | . | . | C | . | . | C | . | T | A | – | – | – | A | A | G | . | . | 11 |
| w-377T | T | . | – | . | . | . | . | . | . | . | C | . | . | C | . | T | A | – | – | – | A | A | G | T | . | 1 |

Ålv, Ljusnan and Dalälven) several variants are present. Only one variant is found in Enningdalsälven and Ljungan, where two and three animals, respectively, were assessed.

Variants y-290G and w-377G are distributed over the entire study area, whereas variant z-242T is restricted to the middle-west catchment areas (see Fig. 8). Interestingly, only these three variants (y-290G, w-377G and z-242T) were found in the west part of Sweden. Variants y-290A, w-377T and z-242G are found in the same catchment areas as their closest allelic counterpart (y-290G, w-377G and z-242T, respectively).

Results of AMOVA (analysis of molecular variance) show more variation within than among populations (59.8% and 40.2% of the variation, respectively).

**Discussion**

**Species distribution**

The four species encountered in Sweden are all considered cosmopolitan (*Ephydatia fluviatilis, Eunapius fragilis*) or widespread in the northern hemisphere (*Ephydatia muelleri, Spongilla lacustris*), with *S. lacustris* having a more boreal distribution (Penney & Racek 1968). The patterns observed in Sweden largely agree with Estonian and Norwegian freshwater sponge diversity. As in Norway (Økland & Økland 1996), the species most commonly encountered was *S. lacustris*, in contrast to Estonia (Lopp et al. 2007) and the Danubean floodplain in Austria (Dröscher & Waringer 2007; Andjus et al. 2017), where *E. fluviatilis* was the most common species. *Ephydatia fluviatilis* is also most common in Denmark (Tendal 1967a) and in Belgium (Richelle-Maurer et al. 1994), albeit with *S. lacustris* almost equally frequent. Curiously, *E. fluviatilis* was not encountered in the freshwater systems sampled, but all specimens collected were found outside river mouths in the Baltic. Arndt (1932) only reported seven sites, all in lakes, and there are also seven localities marked in the Atlas of Pronzato & Manconi (2001), so it is likely it is rare in inland waters, and was missed in our survey. It is also possible that it is present at some sites, but at greater depths than we sampled. However, this seems to be consistent with *S. lacustris* having a more boreal distribution.

As in Norway (Økland & Økland 1996) the present study also found *E. muelleri* to be more common than *E. fluviatilis*, contrary to the case in Belgium, Denmark, Estonia and UK (Tendal 1967a; Richelle-Maurer et al. 1994; Lopp et al. 2007; Evans & Montagnes 2019). Finally, Arndt’s (1932) suggestion
that *Trochospongilla horrida* Weltner, 1893 may be present in Sweden remains unsubstantiated, as no specimen was encountered, and this is also consistent with the lack of reports from neighboring countries.

As in several of the other studies (Tendal 1967a; Lopp *et al.* 2007; Evans & Montagnes 2019), species often co-occurred (Table 2), but as our sampling was not specifically aimed at studying co-occurrence, and sample size was in some cases small, the numbers should not be over-interpreted.

**Spongilla lacustris** phylogeography

Even though we were not successful in obtaining i56 sequences for all the specimens of *S. lacustris*, sequences were obtained from specimens sampled in nine main catchment areas.

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*Fig. 7.* Networks of i56 varieties in Swedish *Spongilla lacustris* (Linnaeus, 1759). Colours indicate catchment area. Seven varieties are designated; z-242T, z-242G, t-24G, y-290G, y-290A, w-377G and w-377T, see Table 3. **A.** PopART integer neighbor-joining network (described in Leigh & Bryant 2015). Pie chart size proportional to the number of specimens found of the variety. Hatches indicate number of substitutions between varieties, sites with indels are excluded; thus, no differences are picked up between z-242T and z-242G. **B.** SplitsTree neighbor network based on HKY85 distances.
Our results show that genetic variation is low, and no clear distribution pattern can be deduced from our data. However, whereas several varieties are generally present in any catchment area, an interesting pattern can be observed from specimens collected in Ljusnan. Data from eight specimens collected in this catchment area shared two varieties: y-290G and y-290A (Fig. 8). In neighbouring catchment areas, other alleles are present, such as w-377G in Ljungan (three specimens) and z-242G and z-242T in Dalälven (six specimens). Thus, gene flow might be restricted.

Network analysis results are congruent with AMOVA; there is more variation within catchment areas (treated as populations) than between them. Thus, no significant structure is observed. As varieties are widespread, the possibility of gene flow between catchment areas cannot be excluded. It thus seems that sponges living in different catchment areas are connected to each other and dispersal might not be severely restricted. Some populations are genetically closer to populations located in the nearest catchment area (such as in eastern Sweden for example), but this pattern is not confirmed for all populations.

Table 4. Differences below diagonal, counting gaps as fifth state, and genetic distances (HKY85) as percentages above diagonal, between the different i56 varieties.

|       | y-290A | y-24G | y-290G | z-242T | z-242G | w-377G | w-377T |
|-------|--------|-------|--------|--------|--------|--------|--------|
| y-290A | –      | 0.45% | 0.22%  | 1.57%  | 1.80%  | 2.51%  | 2.74%  |
| y-24G  | 3      | –     | 0.22%  | 2.02%  | 2.25%  | 2.50%  | 2.73%  |
| y-290G | 2      | 1     | –      | 1.80%  | 2.02%  | 2.27%  | 2.50%  |
| z-242T | 15     | 18    | 17     | –      | 0.22%  | 1.56%  | 1.79%  |
| z-242G | 16     | 19    | 18     | 1      | –      | 1.56%  | 1.79%  |
| w-377G | 23     | 22    | 21     | 13     | 13     | 1      | –      |
| w-377T | 24     | 23    | 22     | 14     | 14     | 1      | –      |

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More studies are needed to draw conclusions on *S. lacustris* phylogeography and potential dispersal. To comprehend sponge distribution, it is fundamental to apprehend gemmulae dispersal. Anemochory and zoochory are often cited as main factors influencing sponge distribution, but were never confirmed as dispersal mechanisms (Frost *et al.* 1982; Manconi & Pronzato 2016). However, wind has little chance to influence gemmulae dispersal. In Sweden, freshwater environments in general do not dry out, and gemmulae fall into the bottom sediments when they detach from their substrate. Nevertheless, gross morphology may enable gemmulae to attach to animals thanks to protruding spicules. The possibility that gemmulae can pass unharmed through the digestive tract of, e.g., waterfowl can’t be ruled out either, and may be an area for further studies. Interestingly, these resting bodies resemble diaspores from the aquatic quillworts *Isoëtes* in shape and size (Korall & Thollesson pers. obs.), and one may speculate that this might be an adaptation to similar dispersal vectors. For lycophytes, it has been observed that zoochory as well as anemochory play a crucial part in spore dispersal (Troia 2016).

The available data cannot discard the higher within catchment area relatedness hypothesis. Therefore, this could be the focus of further study. The development of genetic markers especially designed for species of Spongillidae could provide further insight into sponge distribution and dispersal.
Fig. 8. Presence of i56 sequence varieties in *Spongilla lacustris* (Linnaeus, 1759) at different collecting sites. Black lines on the map represent watersheds between the catchment areas and cross hairs mark the actual sampling site. Seven sequence varieties were found, designated z-242T, z-242G, y-290G, y-290A, y-24G, w-377G and w-377T. Pie charts indicate fraction of the varieties at a site, and circle area corresponds to number of specimens sequenced from the site.
Despite freshwater sponges’ potential environmental value, such as controlling invasive species (Ricciardi 2015), recycling organic matter and habitat building for diverse organisms (studied in marine sponges, see, e.g., Folkers & Rombauts 2019), they are not red-listed and seldom reported in environmental surveys (Manconi & Pronzato 2008). Monitoring freshwater sponge populations is important, as their presence can help in the assessment of environmental quality (Richelle et al. 1995; Dröscher & Waringer 2007).

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Supplementary file
Supp. file 1. List of primer pairs tested in the present study. Dir indicates the primer direction; F, forward and R, reverse. Homolens version is indicated by the number, and the gene family is given by HBG-code. https://doi.org/10.5852/ejt.2022.828.1861.7303