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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 (*cox*I, 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 (Økland & Økland 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 (Økland & Økland 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 (Helgarsviken), 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 $MgCl_2$, whereas a varying concentration of $MgCl_2$ was tried when trying markers for phylogeography. PCR was done in an Applied Biosystem Veriti 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).

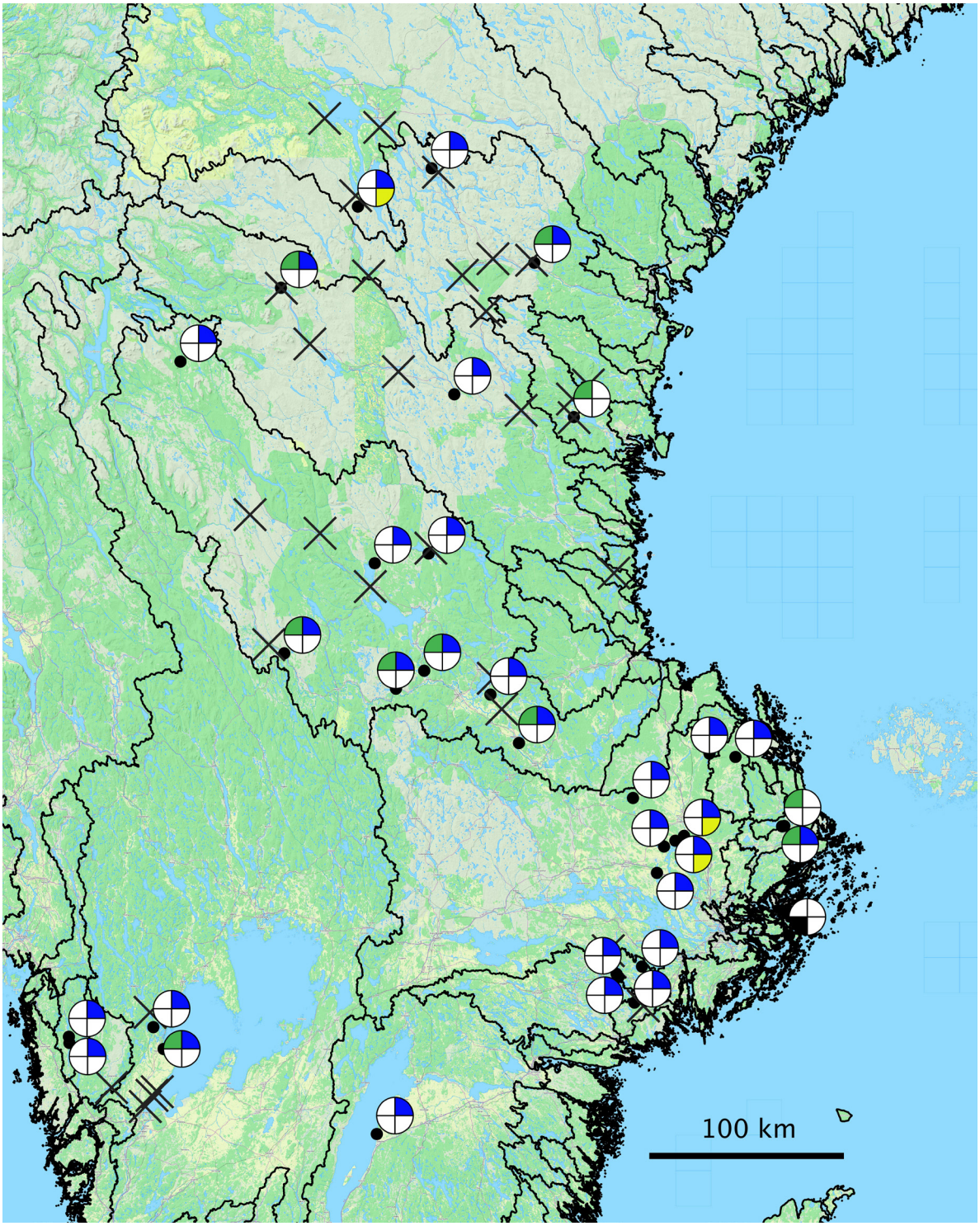

Fig. 1. Sampling sites and species found in main catchment areas in Sweden. Black lines represent watersheds between catchment areas, from the Swedish Meteorological and Hydrological Institute SVAR database (Westman *et al.* 2017). Crosses are sites visited but where no sponge was found, dots are sites 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 *cox*I, 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 *cox*I 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 *cox*I 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, ATPaseβ intron, a *cox*I 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 *cox*I (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 ATPaseβ. 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 @ θ_{anneal} [decreased 1°C/cycle], 1 min @ 73°C), 25 × (40s @ 94°C, 40s @ θ_{anneal}, 1 min @ 72°C), 3 min @ 73°C. The annealing temperature, θ_{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 *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 *cox*I 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 *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* (Lieberkühn, 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

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, Håverud; 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 188110, 188112 • 3 specs; Hammarskogs bath; 59.7636° N, 17.5762° E; 2 Aug. 2017; Chloé Robert and Raquel Pereira leg.; UPSZMC 188122 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.

		Collection site			GenBank accession numbers				
Species	**Specimen ID**	**Designation**	**Coordinates**	**Catchment area**	***coxI***	**28S**	**i56**	**i56 variety**	**Voucher number(s)**
Spongilla lacustris	P059-170830-1	Storsjön, Galhammar, Jmt	62°46.7′ N 14°25.93′ E	Indalsälven					UPSZMC 188161, 188162
	P059-170830-9				OL979196				UPSZMC 188183, 188184
	P059-190702-1	Storsjön, Slandrom, Jmt	63°8.08′ N 14°38.87′ E						UPSZMC 188209
	P059-190702-2								UPSZMC 188210
	P059-190702-3								UPSZMC 188211
	P059-190702-4								UPSZMC 188212
	P059-170829-1	Gissjön, Mpd	62°31.4′ N 16°10.74′ E	Ljungan	OL979206				UPSZMC 188147
	P059-170829-2								UPSZMC 188148
	P059-170829-6	Sundsjön, Jmt	62°57.08′ N 15°9.74′ E				OM105905	w-377G	UPSZMC 188155, 188156
	P059-170829-7						OM105907	w-377G	UPSZMC 188157, 188158
	P059-170829-8						OM105933	w-377G	UPSZMC 188159, 188160
	P059-130831-1	Ängratörn, by Vikstenstorpet, His	61°54.95′ N 15°23.23′ E	Ljusnan					UPSZMC 188092, 188093
	P059-130831-2				OL979184		OM105936	y-290A	UPSZMC 188094, 188095
	P059-130831-3				OL979198	OL985646			UPSZMC 188096, 188097
	P059-170830-11	Hedeviken, Hrj	62°24.5′ N 13°40.18′ E						UPSZMC 188165
	P059-170830-12						OM105912	y-290G	UPSZMC 188166, 188167
	P059-170830-14						OM105904	y-290G	UPSZMC 188170
	P059-170830-15								UPSZMC 188171
	P059-170830-16						OM105919	y-290G	UPSZMC 188172
	P059-170830-17								UPSZMC 188173
	P059-170830-18						OM105901	y-290G	UPSZMC 188174

Table 1 (continued on next six pages). Material examined in the present study.

		Collection site			GenBank accession numbers				
Species	**Specimen ID**	**Designation**	**Coordinates**	**Catchment area**	***coxI***	**28S**	**i56**	**i56 variety**	**Voucher number(s)**
Spongilla lacustris	P059-170830-19						OM105903	y-290G	UPSZMC 188175
	P059-170830-20						OM105932	y-290A	UPSZMC 188176
	P059-170830-21								UPSZMC 188177, 188178
	P059-170830-22								UPSZMC 188179, 188180
	P059-170830-23						OM105924	y-290G	UPSZMC 188181, 188182
	P059-130828-1	Dalälven, Grådö, Dlr	60°14.83′ N 16°1.62′ E	Dalälven		OL985645			UPSZMC 188037, 188038
	P059-130828-2								UPSZMC 188056
	P059-130828-3						OM105921	z-242T	UPSZMC 188059, 188060
	P059-130828-4								UPSZMC 188061
	P059-130828-6	Strands boat club, Dlr	60°29.05′ N 15°44.68′ E						UPSZMC 188064, 188065
	P059-130828-16	Flosjön, boat site, Dlr	60°30.78′ N 14°48.53′ E						UPSZMC 188048, 188049
	P059-130828-17								UPSZMC 188050, 188051
	P059-130828-19								UPSZMC 188054, 188055
	P059-130828-20						OM105913	z-242T	UPSZMC 188057, 188058
	P059-130828-10	S Mojesjön, bathing place, Dlr	60°36.02′ N 15°5.31′ E						UPSZMC 188039, 188040
	P059-130828-11								UPSZMC 188041
	P059-130828-12								UPSZMC 188042
	P059-130828-13								UPSZMC 188043, 188044
	P059-130828-14								UPSZMC 188045
	P059-130828-15								UPSZMC 188046, 188047

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

Species	Specimen ID	Collection site			GenBank accession numbers				
		Designation	Coordinates	Catchment area	*coxI*	28S	i56	i56 variety	Voucher number(s)
Spongilla lacustris	P059-130829-1	Malungs camping, mouth of the river in Västerdalälven Dlr	60°41.08′ N 13°42.14′ E						UPSZMC 188072, 188073
	P059-130829-2								UPSZMC 188074
	P059-130830-1	Orsasjön, at the beach restaurant, Dlr	61°7.12′ N 14°35.91′ E						UPSZMC 188082
	P059-130830-2								UPSZMC 188083
	P059-130830-3						OM105910	y-290G	UPSZMC 188084, 188085
	P059-130830-4								UPSZMC 188086, 188087
	P059-130830-5						OM105899	y-290G	UPSZMC 188088, 188089
	P059-130830-6	Skattungen, Oresjöns boat club, Upp	61°9.89′ N 15°7.96′ E				OM105915	z-242G	UPSZMC 188090
	P059-130830-7						OM105925	z-242T	UPSZMC 188091
	P052-030828-1	Hällsjön, northern resting place, Dlr	62°4.09′ N 12°40.46′ E		OL979188				UPSZMC 188000
	P052-030830-1				OL979195				UPSZMC 188003
	P059-170803-6	Gimo dam, Upp	60°10.72′ N 18°10.31′ E	Olandsån					UPSZMC 188137, 188138
	P059-170803-7						OM105931	y-290G	UPSZMC 188140, 188141
	UP-16-2-1	Dock at Erken-lab, Upp	59°50.12′ N 18°37.99′ E	Norrtäljeån			OM105917	w-377G	UPSZMC 188213, 188214
	P059-170802-13	Krägga mansion, Upp	59°36.18′ N 17°23.64′ E	Norrström					UPSZMC 188114
	P059-170802-11	Viks badplats, Upp	59°44.08′ N 17°27.83′ E						UPSZMC 188110, 188111
	P059-170802-12						OM105902	w-377G	UPSZMC 188112, 188113
	P059-170802-7	Hammarskogs badplats, Upp	59°45.82′ N 17°34.57′ E			OL985663	OM105920	z-242T	UPSZMC 188122
	P059-170802-8				OL979192				UPSZMC 188123
	P059-170802-9								UPSZMC 188124, 188125

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

Species	Specimen ID	Collection site			GenBank accession numbers				
		Designation	Coordinates	Catchment area	*coxI*	28S	i56	i56 variety	Voucher number(s)
Spongilla lacustris	P059-170802-1	Flottsund, Upp	59°47.25′ N 17°39.76′ E		OL979179		OM105923	y-290G	UPSZMC 188107
	P059-170802-2								UPSZMC 188115
	P059-170802-5								UPSZMC 188120
	P059-170802-6								UPSZMC 188121
	P059-140526-1	Siggefora Lake, Upp	59°58.55′ N 17°9.42′ E						UPSZMC 188100, 188101
	P059-140526-2								UPSZMC 188102, 188103
	P059-140526-3								UPSZMC 188104, 188105
	P059-140526-4								UPSZMC 188106
	P059-170803-1	Simbadet, Österbybruk, Upp	60°11.7′ N 17°54.86′ E						UPSZMC 188126, 188127
	P059-170803-2								UPSZMC 188129, 188130
	P059-170803-3						OM105928	w-377G	UPSZMC 188131, 188132
	P059-170803-4								UPSZMC 188133, 188134
	P059-170803-5								UPSZMC 188135, 188136
	P059-130629-1	Likstammen, east Malma, Sdm	58°56.81′ N 17°10.25′ E	Svärtaån			OM105930	y-290G	UPSZMC 188012, 188013
	P059-130629-2								UPSZMC 188015
	P059-130629-3						OM105897	y-290G	UPSZMC 188016
	P059-130629-4					OL985649	OM105922	z-242T	UPSZMC 188017
	P059-130629-5						OM105896	y-290G	UPSZMC 188018
	P059-130630-1	Naten, Stjärnhof bathing place, Sdm	59°4.64′ N 17°0.77′ E		OL979207	OL985655	OM105927	y-290G	UPSZMC 188023
	P059-130630-2					OL985644	OM105908	y-290G	UPSZMC 188029
	P059-130630-3				OL979201		OM105911	w-377G	UPSZMC 188030

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

Species	Specimen ID	Collection site			GenBank accession numbers				
		Designation	Coordinates	Catchment area	*coxI*	28S	i56	i56 variety	Voucher number(s)
Spongilla lacustris	P059-130630-4						OM105900	z-242T	UPSZMC 188031
	P059-130630-5				OL979189	OL985652			UPSZMC 188032
	P059-130630-6					OL985667			UPSZMC 188033
	P059-130630-7					OL985658			UPSZMC 188034
	P059-130630-8					OL985660	OM105934	z-242T	UPSZMC 188035
	P059-130630-9					OL985659	OM105914	w-377G	UPSZMC 188036
	P059-130630-10	Kyrksjön, bathing place, Sdm	59°5.54′ N 16°59.71′ E						UPSZMC 188024
	P059-130630-11								UPSZMC 188025
	P059-130630-12								UPSZMC 188026
	P059-130630-13								UPSZMC 188027
	P059-130630-14								UPSZMC 188028
	P059-130629-10	Klämmingen, Solbacken, Sdm	59°7.84′ N 17°14.72′ E		OL979203	OL985664	OM105926	w-377G	UPSZMC 188014
	P059-130629-6				OL979187	OL985656			UPSZMC 188019
	P059-130629-7				OL979182	OL985670			UPSZMC 188020
	P059-130629-8					OL985648	OM105916	y-24G	UPSZMC 188021
	P059-130629-9				OL979191		OM105909	y-290G	UPSZMC 188022
	P059-171011-1	Vänern, Sunannå Hamn, Mellerud, Dls	58°42.55′ N 12°30.43′ E	Göta Älv					UPSZMC 188195, 188196
	P059-171011-2						OM105935	w-377G	UPSZMC 188197, 188198
	P059-171011-4						OM105898	w-377G	UPSZMC 188199, 188200
	P059-171011-6	Åklång, Dalslands kanal, Håverud, Dls	58°49.28′ N 12°24.37′ E						UPSZMC 188201, 188202
	P059-171011-7								UPSZMC 188203, 188204
	P059-171011-8						OM105906	w-377T	UPSZMC 188205, 188206

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

Species	Specimen ID	Collection site			GenBank accession numbers				
		Designation	Coordinates	Catchment area	*coxI*	28S	i56	i56 variety	Voucher number(s)
Spongilla lacustris	P059-171011-9								UPSZMC 188207, 188208
	P059-171008-1	Bullaresjön, Sundshult, Naverstad, Bhl	58°44.42′ N 11°34.46′ E	Enningdalsälven			OM105929	y-290G	UPSZMC 188185, 188186
	P059-171008-3	S Bullaresjön, Östad, Bhl	58°46.31′ N 11°34.16′ E						UPSZMC 188189, 188190
	P059-171008-4								UPSZMC 188191, 188192
	P059-171008-5				OL979199				UPSZMC 188193, 188194
Eunapius fragilis	P059-170830-10	Storsjön, Galhammar, Jmt	62°46.7′ N 14°25.93′ E	Indalsälven	OL979181				UPSZMC 188163, 188164
	P059-170830-2				OL979200				UPSZMC 188215, 188216
	P059-170830-3				OL979183				UPSZMC 188221, 188222
	P059-170830-4				OL979180	OL985661			UPSZMC 188217, 188218
	P059-170830-5				OL979202	OL985653			UPSZMC 188223, 188224
	P059-170830-6				OL979209				UPSZMC 188225, 188226
	P059-170830-7				OL979185				UPSZMC 188227, 188228
	P059-170830-8				OL979210	OL985651			UPSZMC 188219, 188220
	P059-170802-10	Hammarskogs badplats, Upp	59°45.82′ N 17°34.57′ E	Norrström	OL979193	OL985650			UPSZMC 188108, 188109
	P059-170802-3	Flottsund, Upp	59°47.25′ N 17°39.76′ E		OL979205				UPSZMC 188116, 188117
	P059-170802-4				OL979197				UPSZMC 188118, 188119
Ephydatia muelleri	P059-170829-3	Gissjön, Mpd	62°31.4′ N 16°10.74′ E	Ljungan	OL979204	OL985642			UPSZMC 188149, 188150
	P059-170829-4					OL985669			UPSZMC 188151, 188152
	P059-170829-5				OL979190	OL985668			UPSZMC 188153, 188154

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

Species	Specimen ID	Collection site			GenBank accession numbers				
		Designation	Coordinates	Catchment area	*coxI*	28S	i56	i56 variety	Voucher number(s)
Ephydatia muelleri	P059-170828-1	Delsbo, Södra Dellensjön, Hls	61°48.58′ N 16°34.24′ E	Delångersån					UPSZMC 188143, 188144
	P059-170828-2				OL979186	OL985654			UPSZMC 188145, 188146
	P059-170830-13	Hedeviken, Hrj	62°24.5′ N 13°40.18′ E	Ljusnan	OL979194	OL985657			UPSZMC 188168, 188169
	P059-130828-5	Dalälven, Grådö, Dlr	60°14.83′ N 16°1.62′ E	Dalälven		OL985662			UPSZMC 188062, 188063
	P059-130828-18	Flosjön, boat site, Dlr	60°30.78′ N 14°48.53′ E						UPSZMC 188052, 188053
	P059-130828-7	S Mojesjön, bathing place, Dlr	60°36.02′ N 15°5.31′ E			OL985647			UPSZMC 188066, 188067
	P059-130828-8					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, 188079
	P059-130829-5								UPSZMC 188080, 188081
	UP-16-2-2	Dock at Erken-lab, Upp	59°50.12′ N 18°37.99′ E	Norrtäljeån					UPSZMC 188244, 188244
	UP-16-1-2	Badplatsen Svanberga: Erken - S bathing place drainage pipe, Upp	59°50.22′ N 18°39.35′ E						UPSZMC 188242, 188243
	P059-171011-3	Vänern, Sunannå Hamn, Mellerud, Dls	58°42.55′ N 12°30.43′ E	Göta Älv					UPSZMC 188237, 188238
	P059-171011-5								UPSZMC 188239, 188240
Ephydatia fluviatilis	P059-170902-1	Helgarsviken, Sth	59°17.34′ N 18°42.19′ E	Östersjön (the Baltic)	OL979208	OL985643			UPSZMC 188229, 188230
	P059-170902-2								UPSZMC 188231, 188232
	P059-170902-3								UPSZMC 188233, 188234
	P059-211231-1								PENDING

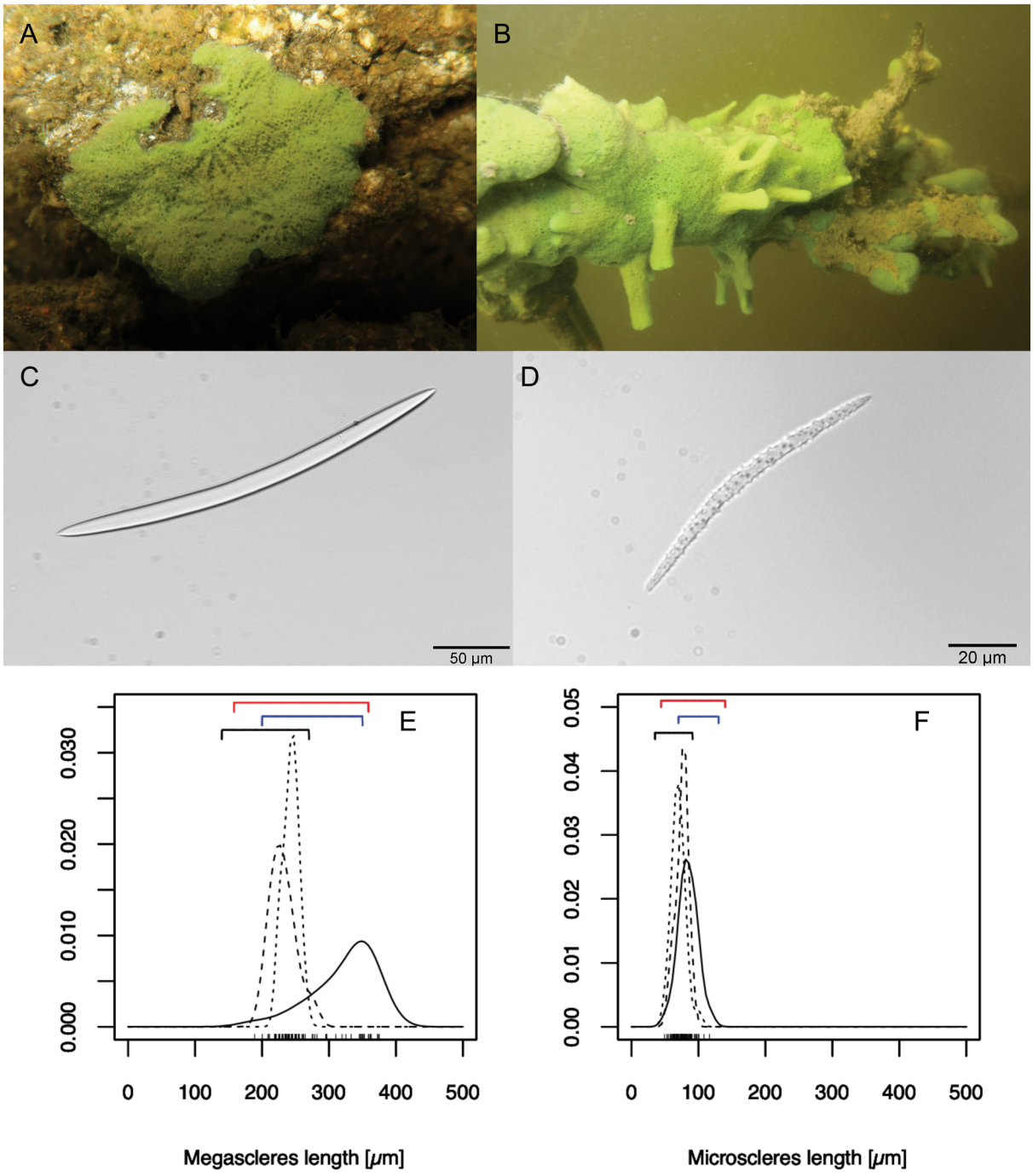

Fig. 2. *Spongilla lacustris* (Linnaeus, 1759). **A–B**. Habitus. **A**. Specimen growing under a pontoon (P059-171011-6). **B**. Specimen growing on anchor chain, with finger–like projections (P059-170802-48). **C–D**. Spiculae. **C**. Megasclere (P059-171008-4). **D**. Microsclere (P059-171008-1). **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

18.6331° E; 30 May 2016; Raquel Pereira leg.; UPSZMC 188213 • 4 specs; Lake Siggeforasjön; 59.9759° N, 17.157° E; 26 May 2014; Tove Fällman leg.; UPSZMC 188100, 188102, 188104, 188106 • 2 specs; Gimo damm; 60.1786° N, 18.1718° E; 3 Aug. 2017; Chloé Robert and Raquel Pereira leg.; UPSZMC 188137, 188140 • 5 specs; Simbadet, Österbybruk; 60.195° N, 17.9143° E; 3 Aug. 2017; Chloé Robert and Raquel Pereira leg.; UPSZMC 188126, 188129, 188131, 188133, 188135.– **Dalarna** • 4 specs; Dalälven, Grådö; 60.2471° N, 16.027° E; 28 Aug. 2013; Mikael Thollesson leg.; UPSZMC 188037, 188056, 188059, 188061 • 1 spec.; Lake Viksjön, Strands boat club; 60.4841° N, 15.7447° E; 28 Aug. 2013; Mikael Thollesson leg.; UPSZMC 188064 • 4 specs; Lake Flosjön, harbour, Dala-Floda; 60.513° N, 14.8089° E; 28 Aug. 2013; Mikael Thollesson leg.; UPSZMC 188048, 188050, 188054, 188057 • 6 specs; Lake S Mojesjön, bath; 60.6003° N, 15.0885° E; 28 Aug. 2013; Mikael Thollesson leg.; UPSZMC 188039, 188041 to 188043, 188045, 188046 • 2 specs; Malungs camping, outlet into Västerdalälven; 60.6847° N, 13.7023° E; 29 Aug. 2013; Mikael Thollesson leg.; UPSZMC 188072, 188075 • 5 specs; Lake Orsasjön, Strandrestaurangen; 61.1186° N, 14.5985° E; 30 Aug. 2013; Mikael Thollesson leg.; UPSZMC 188082 to 188084, 188086, 188088 • 2 specs; Lake Skattungen, Oresjöns boat club; 61.1649° N, 15.1327° E; 30 Aug. 2013; Mikael Thollesson leg.; UPSZMC 188090, 188091 • 2 specs; Lake Hällsjön, northern rest stop; 62.0681° N, 12.6744° E; 28 Aug. 2003; Mikael Thollesson leg.; UPSZMC 188000, 188003. – **Hälsingland** • 3 specs; Lake Ängratörn, Vikstenstorpet; 61.9159° N, 15.3872° E; 31 Aug. 2013; Mikael Thollesson leg.; UPSZMC 188092, 188094, 188096. – **Härjedalen** • 12 specs; Hedeviken; 62.4083° N, 13.6697° E; 30 Aug. 2017; Chloé Robert and Raquel Pereira leg.; UPSZMC 188165, 188166, 188170 to 188177, 188179, 188181. – **Medelpad** • 2 specs; Lake Gissjön; 62.5233° N, 16.179° E; 29 Aug. 2017; Chloé Robert and Raquel Pereira leg.; UPSZMC 188147, 188148. – **Jämtland** • 2 specs; Lake Storsjön, Galhammar; 62.7784° N, 14.4321° E; 30 Aug. 2017; Chloé Robert and Raquel Pereira leg.; UPSZMC 188161, 188183 • 3 specs; Lake Sundsjön; 62.9513° N, 15.1623° E; 29 Aug. 2017; Chloé Robert and Raquel Pereira leg.; UPSZMC 188155, 188157, 188159 • 4 specs; Lake Storsjön, Slandrom; 63.1347° N, 14.6478° E; 29 Aug. 2017; Chloé Robert and Raquel Pereira leg.; UPSZMC 188209 to 188212.

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.

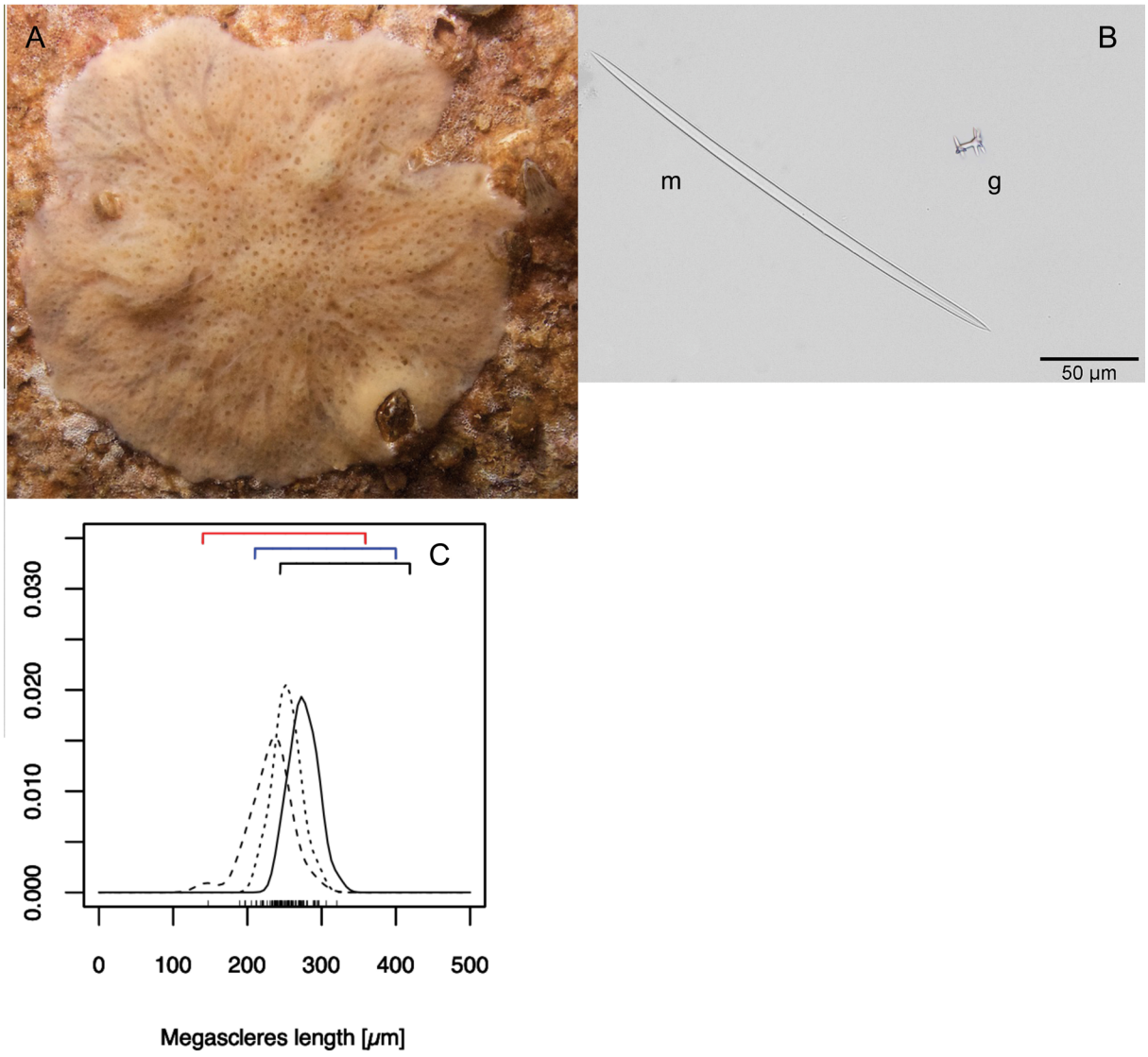

Fig. 3. *Ephydatia fluviatilis* (Linnaeus, 1759). **A**. Habitus (P059-211231-1). **B**. Spiculae, m = megasclere (P059-170902-2), g = gemmulosclere (P059-170902-1). **C**. Estimated megasclere size distribution within and between specimens, P059-170902-1 (solid line), P059-170902-2 (dashed), P059-170902-3 (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

Spongia 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 megasclers 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 mülleri Liberkühn, 1856: 510.

Material examined (18 specimens, Table 1)

SWEDEN – **Dalsland** • 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, Grådö; 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 Dellensjön; 61.8097° N, 16.5706° E; 28 Aug. 2017; Raquel Pereira leg.; UPSZMC 188143, 188145. – **Härjedalen** • 1 spec.; Hedeviken; 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

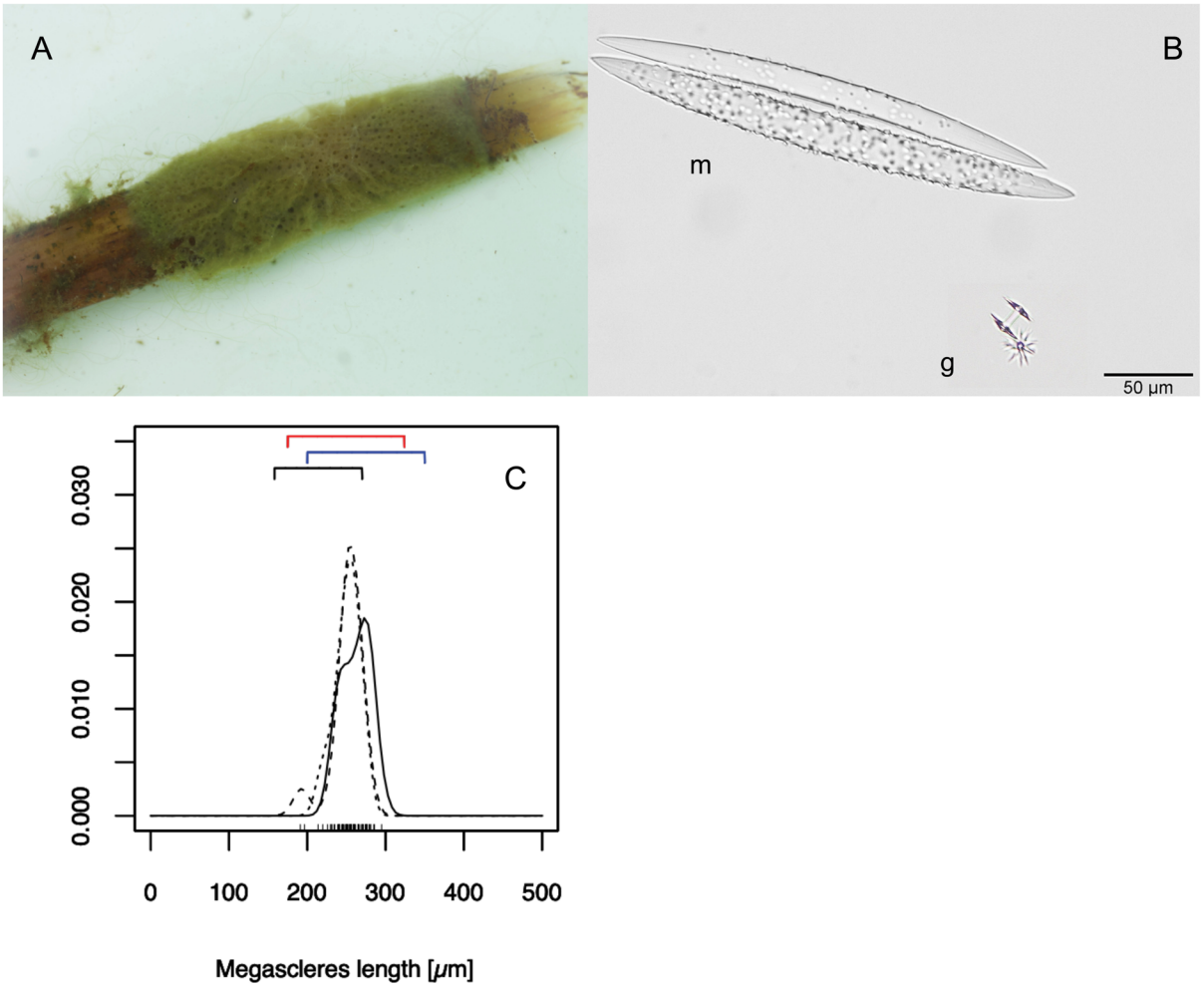

Fig. 4. *Ephydatia muelleri* (Lieberkühn, 1856). **A**. Habitus (P059-170829-4). **B**. Spiculae, m = megascleres (P059-170830-13), g = gemmuloscleres (P059-130828-18). **C**. Estimated megasclere size distribution within and between specimens, P059-130829-3 (solid line), P059-170830-13 (dashed) and 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 *cox*I 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 $MgCl_2$ 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.

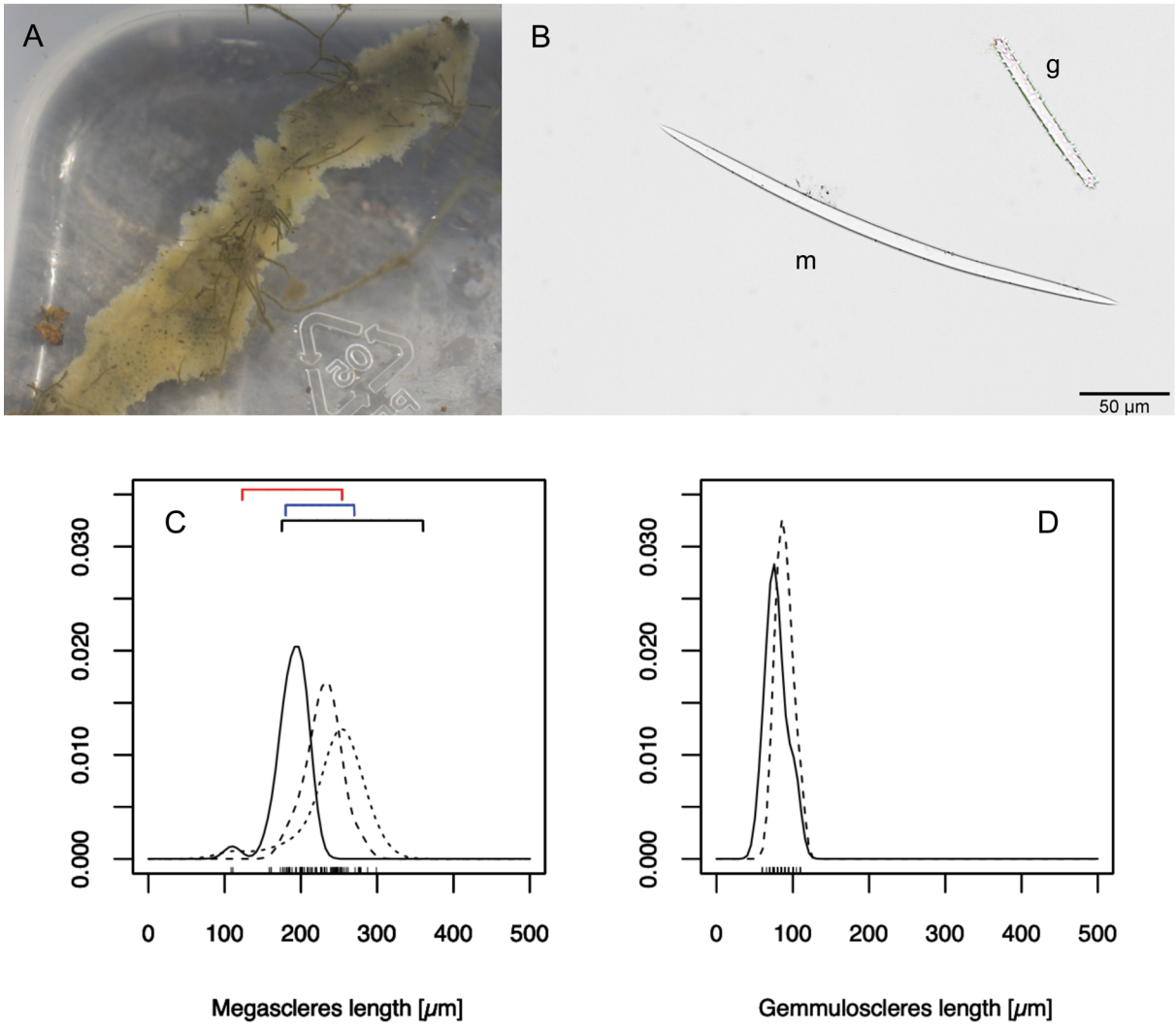

Fig. 5. *Eunapius fragilis* (Leidy, 1851). **A**. Habitus (P059-170829-4). **B**. Spiculae, m = megasclere (P059-170830-13), g = gemmulosclere (P059-170902-1). **C–D**. Estimated spicula size distribution within and between specimens, P059-170802-10 (solid line), P059-170830-2 (dashed) and P059-170830-4 (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.

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

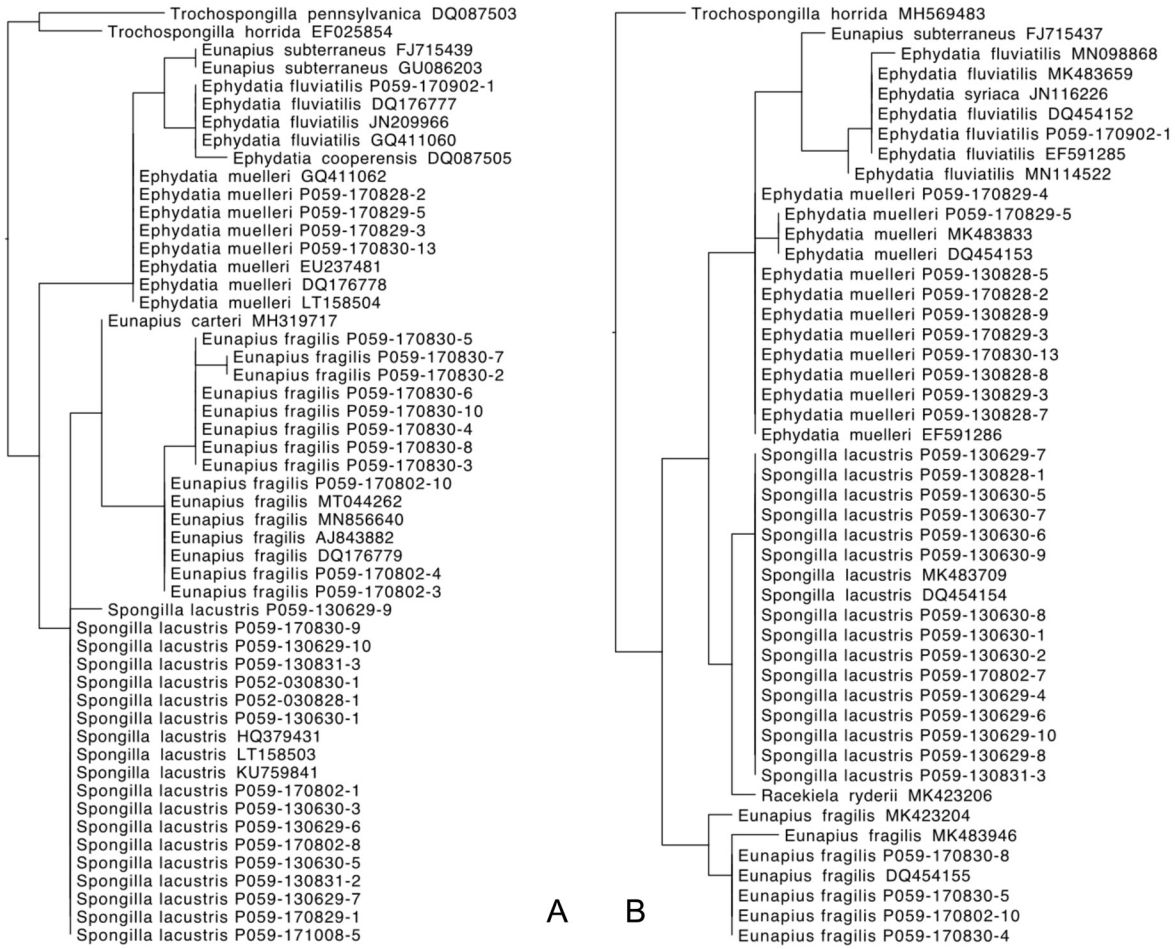

Fig. 6. Neighbor-joining trees based on HKY85 distances. **A**. *coxI* dataset. **B**. 28S dataset. Sequences with a specimen field number (starting with P052- or P059-) are from the present study, remaining sequences are from GenBank with the accession number in the label.

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	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	A	T	G	C	T	G	G	T	C	G	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*

Älv, 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.

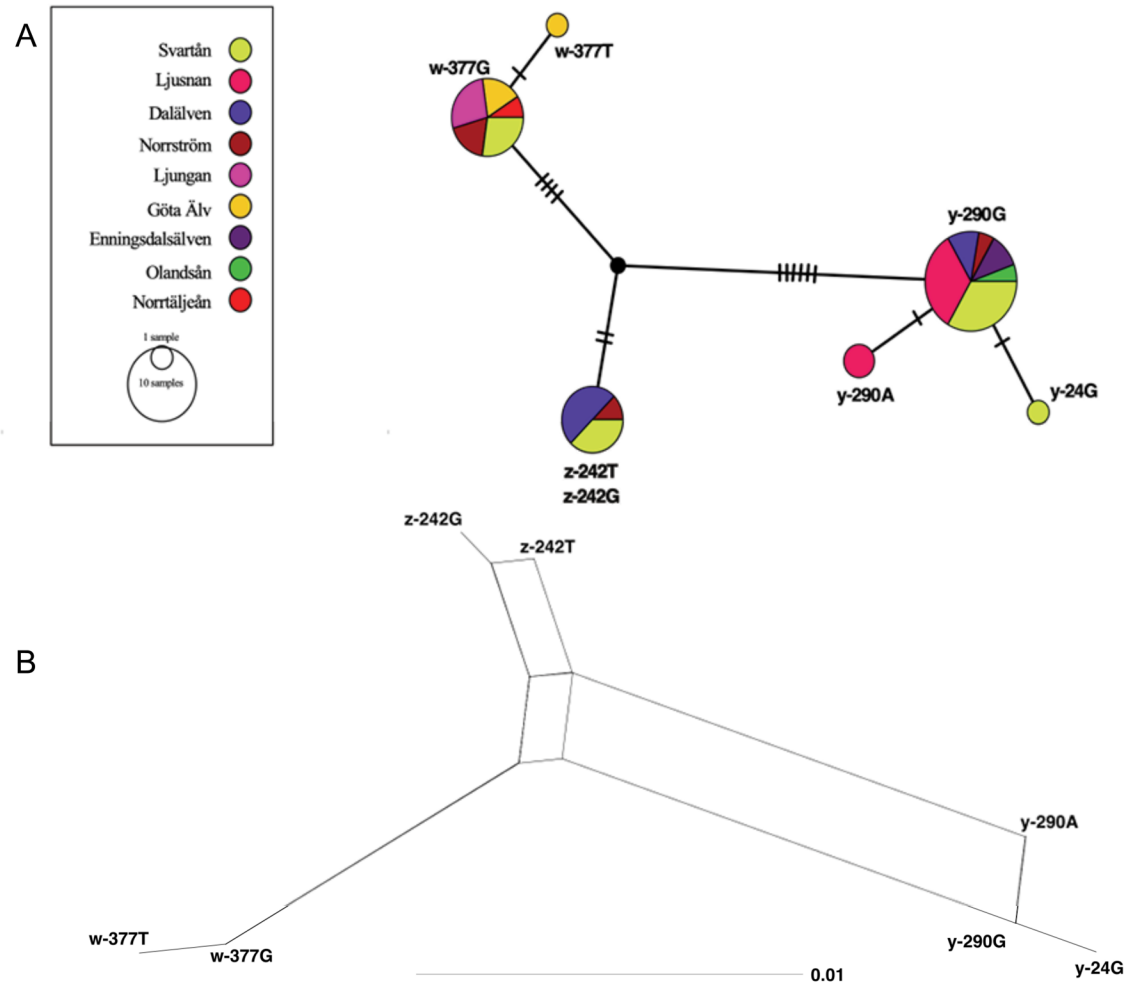

Fig. 7. Networks of i56 varieties in Swedish *Spongilla lacustris* (Linnaeus, 1759). Colours indicate catchment area. Seven varieties are designated; z-242T, z-242G, t-24G, y-290G, y-290A, w-377G and w-377T, see Table 3. **A**. PopART integer neighbor-joining network (described in Leigh & Bryant 2015). 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.

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	–	0.22%
w-377T	24	23	22	14	14	1	–

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.

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.

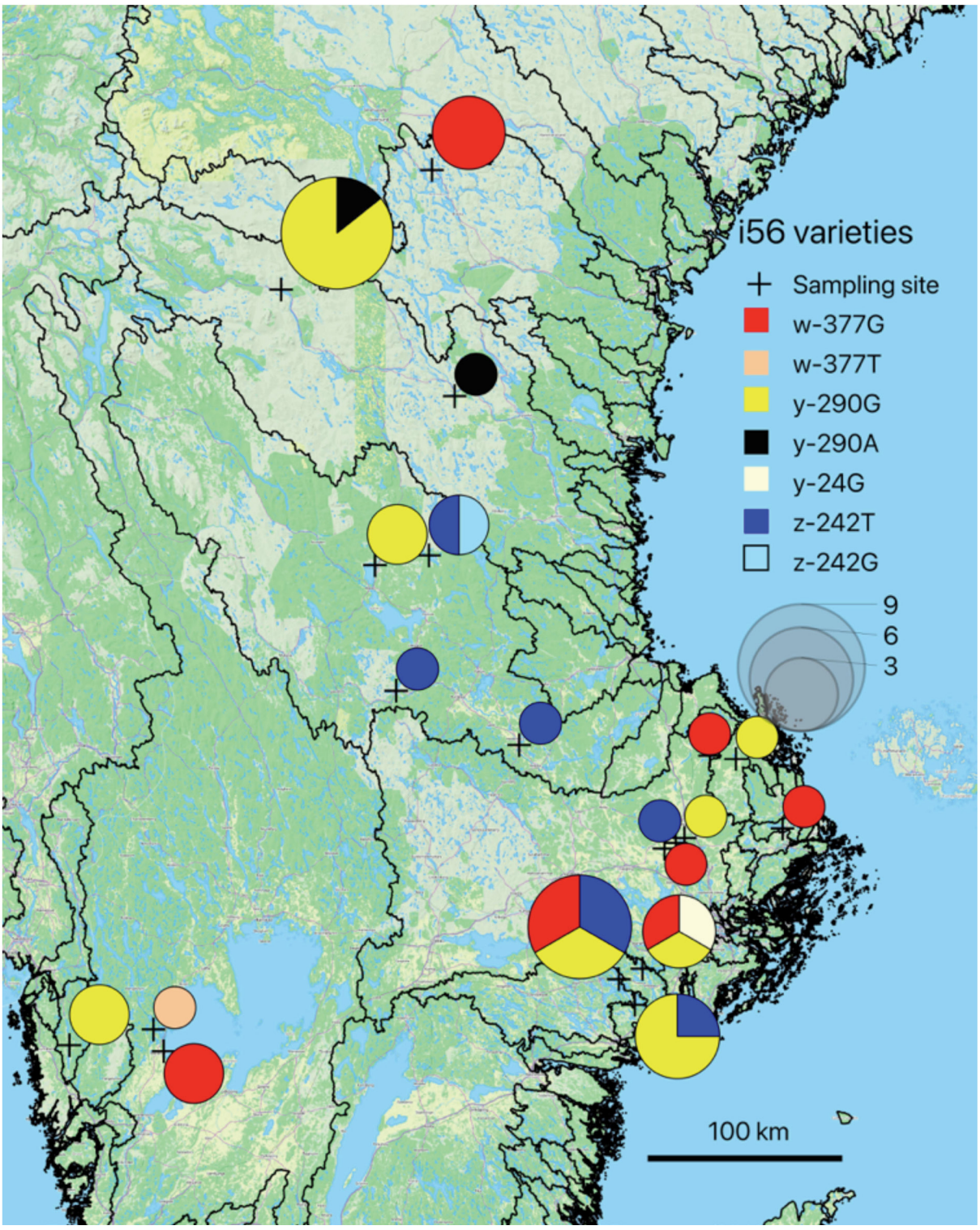

Fig. 8. Presence of i56 sequence varieties in *Spongilla lacustris* (Linnaeus, 1759) at different collecting sites. Black lines on the map represent watersheds between the catchment areas and cross hairs mark the actual sampling site. Seven sequence varieties were found, designated z-242T, z-242G, y-290G, 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