1   Corresponding Author:

2   Stephen H Kolomyjec, College of Science and the Environment, Lake Superior State

3   University, 650 W Easterday Ave., Sault Sainte Marie, MI 49783, USA, +1 906-635-2147,

4   E-mail: skolomyjec@lssu.edu

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11   **Phylogenetic analysis of Michigan's freshwater sponges (Porifera, Spongillidae) using**

12   **extended COI mtDNA sequences**

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14   STEPHEN H. KOLOMYJEC[1], ROGER A. WILLFORD[1, 2], & THE FALL 2019

15   GENETICS CLASS[1]

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17   [1]College of Science and the Environment, Lake Superior State University, 650 W Easterday

18   Ave., Sault Sainte Marie, MI 49783, USA

19   [2]Undergraduate Research Student

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28   Running Title:

29   Phylogenetics of Michigan's freshwater sponges

30   Kolomyjec *et al.*

31 **Abstract**

32 Kolomyjec *et al.* (2020). Phylogenetic analysis of Michigan's freshwater sponges (Porifera,

33 Spongillidae) using extended COI mtDNA sequences. – *Zoologica Scripta*, 00, 000-000.

34 The phylogenetic relationships of eight species of freshwater sponges sampled throughout

35 the State of Michigan in the North American Great Lakes region were examined as part of a

36 Course-based Undergraduate Research Experience (CURE). An extended version of the

37 standard cytochrome oxidase subunit I (COI) metazoan DNA was used for sequencing and

38 Bayesian phylogenetic inference. The extended gene region (COI-ext) produces a 1,200 bp

39 amplicon instead of the standard 640 bp fragment which compensates for the standard

40 amplicon's low informatics value in Phylum Porifera. The species examined clustered into

41 strongly supported monophyletic species groups within the family *Spongillidae*. This study

42 represents the first look at the phylogenetic relationships of freshwater sponges in the Great

43 Lakes Region.

44 *Per Kolomyjec, College of Science and the Environment, Lake Superior State University,*

45 *650 W Easterday Ave., Sault Sainte Marie, MI 49783, USA. E-mail skolomyjec@lssu.edu*

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Introduction

Freshwater sponges are the pinnacle of the weird and wonderful. As a basal animal that lack some of the important aspects of the eumetazoans such a true tissues or organs, sponges are of keen interest to evolutionary biologists studying the origin and evolution of the multicellular animal body form (e.g. Nielsen 2008; Borchiellini *et al.* 2001). As sessile filter feeders, sponges provide a wide range of ecological roles and services from filtering water to providing habitat and refuge to countless organisms, even contributing to primary production in their local ecosystems through symbiosis with a wide variety of autotrophic organisms (Reiswig et al 2010; Manconi & Pronzato 2016). There is even the potential for extensive bioprospecting for the novel secondary metabolites formed by sponges and their extensive microbiome (e.g. Laport & Santos 2009; Simmons *et al.* 2005; Balasubramanian *et al.* 2018). Despite all of this, and the volume of research focused on marine sponges, most people are not even aware that freshwater sponges exist, and they remain relatively understudied.

The field of freshwater sponge research is a relatively small global community continually working towards an improved understanding of distribution and taxonomy at the continental to global scale (e.g. Manconi & Pronzato 2008, 2002, 2015; Reiswig *et al.* 2010; Ricciardi & Reiswig 1993; Volkmer-Ribeiro & Travest 1987). Of particular regional importance to the authors is the doctoral thesis and resulting publications of M. C. Old (1930, 1931) which detail the last and most complete state-wide survey of freshwater sponge diversity in Michigan. This past study places the reported number of species found in Michigan waters at 12 species within 10 genera (Old 1931), with a total of 31 species within 14 genera currently reported for continental North America (Manconi & Pronzato 2015).

The vast majority of research has been carried out using morphological features to determine species identification and taxonomy of the freshwater sponges. To date few studies have used molecular characters to explore freshwater sponge systematics and taxonomy (Addis & Peterson 2005). This dearth of information hampers research in freshwater biodiversity and conservation. Currently, identification of freshwater sponge species to the genus or species level requires the careful examination of microscopic traits

77   based on spicule and gemmule morphology (e.g. Old 1931; Reswig *et al.* 2010; Manconi &

78   Pronzato 2002). The seasonality of gemmules in many species and the high degree of

79   phenotypic plasticity observed in freshwater sponges leads to the ability to meaningfully

80   identify and quantify sponges to beyond the effort/benefit threshold of most freshwater

81   research endeavors. This commonly leads to sponges only being included and quantified at

82   the phylum level (MI DEQ 2000). This obviously renders a non-representative view of any

83   system's actual biodiversity and conservation needs.

84   A molecular approach to species identification, such as DNA barcoding, in

85   biological research has the potential to alleviate this barrier and opens the possibility of

86   species detection using aquatic eDNA approaches (e.g. Pilliod *et al.* 2013). However, this

87   requires adequate baseline data which as mentioned is lacking for freshwater sponges. For

88   example, at the time of this project GenBank (Sayers *et al.* 2019) only contained COI

89   (Cytochrome Oxidase subunit I) sequences, commonly used for DNA barcoding of

90   animals, for five of Michigan's 12 species of sponges, with only three of those species

91   having more than a single DNA record.

92   These gaps in the available data became evident during the first large scale resurvey

93   of Michigan's freshwater sponges in over 80 years (Old 1931) and the efforts reported in

94   this study were focused on using the COI gene as a molecular marker for exploring the

95   phylogenetic relationships of Michigan's freshwater sponges and expanding the range of

96   publicly available DNA sequences available to aide future research.

97

98   **Materials and Methods**

99   ***Course-based Undergraduate Research Experience (CURE)***

100   This research was conducted as a course-based undergraduate research experience (CURE),

101   which is an approach to integrating authentic research into the undergraduate curriculum in

102   a manner that allows participation of a large number of students simultaneously (e.g.

103   (Auchincloss *et al*. 2014; Rowland *et al*. 2012). This CURE was integrated into Dr.

104   Kolomyjec's 200-level genetics class in the fall 2019 semester at Lake Superior State

105   University as an extension of the course's regular DNA barcoding lab. There were 51

106   students actively involved across three lab sections. Individual students were tasked with

107   DNA isolation, PCR and sequencing prep, sequence trimming and quality control, DNA

108   based specimen identification (a.k.a. DNA barcoding) when possible, and initial phylogeny

109   construction.

110

111   ***Sponge samples***

112   Fifty-one 70% ethanol preserved sponge specimens were semi-randomly selected from

113   samples collected during a recent statewide survey (Willford & Kolomyjec unpublished).

114   The only criteria for selection was that the individual specimen was large enough for

115   students to subsample without difficult or risk of destroying the voucher specimen. The

116   samples selected represented 17 of the 44 8-digit HUCs (Hydrologic Unit Codes) sampled

117   by Willford & Kolomyjec (2019; unpublished).

118

119   ***Taxonomic identification***

120   Species level identification of freshwater sponges requires careful examination of the

121   microscopic morphology of the hard-skeletal elements (spicules) and over-wintering

122   structures (gemmules). The protocol used was modified from that of Old (1931).

123   Subsamples of the selected specimens were placed in test tubes, dried overnight in a drying

124   oven to remove all traces of ethanol and prevent an explosive reaction with the nitric acid

125   used during tissue digestion. Tissue digestion was performed with concentrated nitric acid

126   (HNO_3) on a 108° C heat block in a fume hood until soft tissues of the sponge were

127   completely dissolved. Nitric acid was then diluted with an equal volume of double-distilled

128   water (ddH_2O). After complete sedimentation of the spicules (about 10-15 minutes) as

129   much liquid as possible was removed from the tube with a disposable transfer pipette. Fresh

130   ddH_2O was added and gently triturated with a new pipette to wash the spicules. Once again,

131   the liquid was removed after sedimentation. The wash process was repeated at least 3 times.

132   After the final wash, the pellet of spicules was resuspended in fresh ddH_2O. A drop of

133   suspended spicules was placed on a standard microscope slide and dried on the same heat

134   block used during tissue digestion. Finally, slides were cover-slipped using a UV cured

135   mounting medium (EUKITT).

136 Species were independently identified by each of the authors using Old (1931), Reswig *et*

137 *al.* (2010), and Manconi & Pronzato (2002, 2016) as primary references. Any IDs in

138 disagreement were compared, reexamined, and resolved together. Genera specialist were

139 consulted when additional confirmation was required.

140

141 *Nucleic acid extraction*

142 Using aseptic techniques, students removed a portion of tissue approximately 3 mm x 3 mm

143 from their assigned specimen for DNA isolation. DNA was isolated using a modified

144 guanidine thiocyanate/silica resin extraction procedure (Cold Spring Harbor 2018). After

145 samples were blot dried with lab tissues, they were placed in 1.5 ml microcentrifuge tubes

146 with 300 µl 6M guanidine thiocyanate using disposable micro-pestles. The resultant slurry

147 was incubated at 65° C for 10 min then centrifuged at 13,000 g for one minute to pellet out

148 cell debris. 150 µl of the resulting supernatant was transferred to a clean 1.5 ml tube along

149 with 3 µl of silica resin (aqueous 325 mesh SiO_2, 50% w/v). This new tube was vortexed

150 and incubated for 10 minutes at 57° C to bind silica and DNA. This was then pelleted by

151 centrifugation at 13,000 g for 30 seconds. The supernatant was discarded, and the pellet

152 washed twice with a wash buffer consisting of 1:1 mix of TNE buffer (1 M Tris (pH 7.4), 5

153 M NaCl, 0.5 M EDTA) and 100% Ethanol. The washed pellet was eluted with 100 µl

154 Molecular Grade H_2O. The unbound silica was pelleted out and the resultant supernatant

155 transferred to a new tube for storage and downstream use.

156 DNA quality and quantity were estimated via spectrophotometry on a Bio-Spec Nano

157 (SHIMADZU).

158

159 *Polymerase chain reaction and sequencing*

160 As the standard 640 bp animal barcoding fragment (Folmer *et al.* 1994) of the Cytochrome

161 Oxidase I gene (COI) gene has been demonstrated to be of low informatics value in the

162 sponges (Erpenbeck *et al.* 2006), a 1,200 bp extended version of the gene was amplified.

163 This extended COI fragment was amplified using the dgLCO1490 (5'-GGT CAA CAA

164 ATC ATA AAG AYA TYG G-3') and COI-R1 (5'-TGT TGR GGG AAA AAR GTT AAA

165 TT-3') degenerate primers (synthesized by INVITROGEN) from Meyer *et al.* (2005) and Rot

166   *et al.* (2016) respectively. PCR reactions were performed in a 55 µl volumes containing: 25

167   µl Hot Start PCR-to-Gel Master Mix (VWR brand); 2 µl of primer mix (25 pmol each of

168   forward and reverse primer); 5 µl template DNA; 23 µl PCR grade H$_2$O. The thermal

169   cycling regime included an initial denaturation phase of 95° C for 5 min followed by 30

170   cycles of: 40 sec denaturation at 95° C; 40 sec annealing 55°C; followed by 60 sec

171   elongation at 72° C; and a final elongation at 72° C for 5 min. PCR success was checked on

172   a standard 2% agarose gel visualized with GelRed nucleic acid stain (BIOTIUM).

173   Sequencing of successful PCR product was performed by the Psomagen Inc. (formerly

174   MacrogenUSA) sequencing core using their LCO1490 universal primer.

175

176   ***Phylogenetic analyses***

177   A set of reference sequences (Table 1) was obtained from GenBank (Sayers *et al.* 2019) for

178   comparison to the experimental samples. The marine demosponge *Tethya aurantium* was

179   selected to serve as the outgroup for analysis. All sequences were trimmed, checked by

180   amino acid reading frames to avoid frameshift errors in alignment, and finally aligned using

181   BioEdit Version 7.0.5.3 (Hall 1999).

182

183   The program BEAST (v1.10.4; Suchard *et al*. 2018) was used for the Bayesian inference of

184   phylogenetic relationships. The default HKY substitution model was utilized as it produced

185   the best Bayesian information criterion score (BIC) in jModelTest v2.1.10 (Darriba *et al.*

186   2012). Markov chain Monte Carlo (MCMC) analyses were run for 1 billion generations

187   (burn-in 200 million generations). BEAST's TreeAnnotator tool was used to estimate the

188   posterior probability of inferred phylogenetic clades via the generation of a maximum clade

189   credibility (MCC) summary tree. Trees were visualized using BEAST's FigTree (v1.4.4)

190   tool and Archaeopteryx (v0.9928; Han & Zmasek 2009).

191

192 **Results**

193 ***Samples and sequencing***

194 Students successfully isolated DNA from 51 freshwater sponge tissue samples. Of those 51

195 DNA isolates, 45 samples were successfully amplified via PCR of the COI locus. PCR

196 product from three additional samples of interest, generated by the class instructor (S

197 Kolomyjec) were included and 48 samples in total were sent off for Sanger sequencing. Of

198 those 48 samples, 42 yielded high-quality sequence for downstream analysis.

199 While awaiting sequencing, authors Willford and Kolomyjec carried out

200 independent morphological identifications of all specimens included in this study. Three

201 samples (K19, K34, and K39) were initially listed as unknown due to a lack of gemmules

202 which prevented conclusive differentiation between *Eunapius fragilis* and *Pottsiella*

203 *aspinosa*. Identity of these samples was later confirmed via localization within the

204 generated COI phylogeny.

205

206 ***Extended Cytochrome Oxidase Subunit I (COI) mtDNA***

207 All COI DNA sequences generated and analyzed in this study have been submitted to

208 GenBank (Supplemental Table S1). Eight out of the twelve species reported to occur in

209 Michigan (OLD 1931, 1932) were represented in this study. This adds COI sequences for

210 three previously unrepresented species to the GenBank database: *Duosclera mackayi;*

211 *Heteromeyenia baileyi; and Pottsiella aspinosa*.

212 When examining the maximum clade credibility (MCC) summary tree (Figure 1 &

213 Supplemental Figure S2) generated by Beast's Tree Annotator, 100% of samples clustered

214 into monophyletic clades representing their species. These species clades were, in turn,

215 supported by moderate to high posterior probability values (0.87-1.00) (Figure 1). Sister

216 clades that support close relationships between species that have traditionally placed in

217 close proximity due to morphological similarities are also present.

218

Discussion

Phylogenetic Relationships of Michigan's Freshwater Sponges

Analysis of an extended version of COI mtDNA (Erpenbeck *et al.* 2005) has provided a clearer phylogenetic interpretation of the relationships between freshwater sponge species than previous studies that utilized the standard COI segment (*e.g.* Addis & Peterson 2005; Meixner *et al.* 2007).This has allowed the authors to examine the interrelationships of Michigan's freshwater sponges with a hereto unachieved clarity while still focusing on the region of DNA commonly used for DNA barcoding based species identification.

All species included in this study cleanly separated into clades matching their taxonomic identity (Figure 1). In the case of *Trochospongilla pennsyanica, Eunapius fragilis, Ephydatia fluviatilis, Ephydatia muelleri,* and *Spongilla lacustris* these clades included the appropriate species-specific reference sequences from GenBank. Intrageneric relationships are also maintained. For example, the two *Ephydatia* species present resolve as sister clades with a posterior probability of 1.00. In turn, the inclusive *Ephydatia* clade is sister to a larger clade containing *S. lacustris* and *Duosclera mackay*i. Interestingly enough *D. mackayi* was originally included within the *Spongilla* genera until Reiswig and Ricciardi (1993) placed it in the monotypic genera *Duosclera* on the basis of extensively unique morphology.

We were able to comfortably assign the three samples that were inconclusively identified via standard light microscopy (K19, K34, and K39) to the *Eunapius fragilis* clade based on their unambiguous phylogenetic placement (S2). Considering the difficulty inherent in identifying freshwater sponges to the species level, this is no minor accomplishment. Setting aside phenotypic variation that often results from the same species of sponge growing under slightly different ecological variation, not all individual sponges will produce all the structures required for definitive identification at all stages of their life cycle. This is a well-established issue in freshwater sponge research (Reiswig et al 2010; Manconi & Pronzato 2016) that can lead to the necessity of repeated sampling across both time and space to resolve the identity of a single species of freshwater sponge within a body of water. Considering the time and monetary expenses of fieldwork in general, the development and validation of supplementary tools such a DNA based identification and

249 verification is critical to the consistent and reliable reporting of species level biodiversity.

250 This study represents a major step forward in refining these tools for freshwater sponge

251 research. While the primary focus of this study was regionally specific to Michigan and the

252 Great Lakes region, many of the species present have a wider, or even global, distribution.

253 While this study provides vital reference sequences and helps to expand the

254 knowledge of the relationships of the freshwater sponges in Michigan and to a lesser extent

255 globally, there is much to be done within the field as a whole. Our research group will

256 continue to work on expanding the sequence-based representation of regional species but to

257 truly understand the relationships across all freshwater sponges a great deal more work

258 needs to be done at the continental and global scale. This work should utilize additional

259 genes or ideally whole genome comparison for hierarchical reconstruction of

260 hydrologically influenced population structure and true global phylogeny.

261

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279   continually supporting student-faculty research collaboration.

280   Finally, we would like to support experiment.com and all of the generous donors

281   that helped fund the field collection of our freshwater sponge samples.

282

283

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371

Table 1 - COI reference sequences from genbank.

Species	Ascension #
Ephydatia fluviatilis	JF340437.1
Ephydatia muelleri	DQ176778.1
Eunapius fragilis	DQ176779.1
Spongilla lacustris	MH985292.1
Tethya aurantium*	EF584565.1
Trochospongilla pennsylanica	DQ087503.1

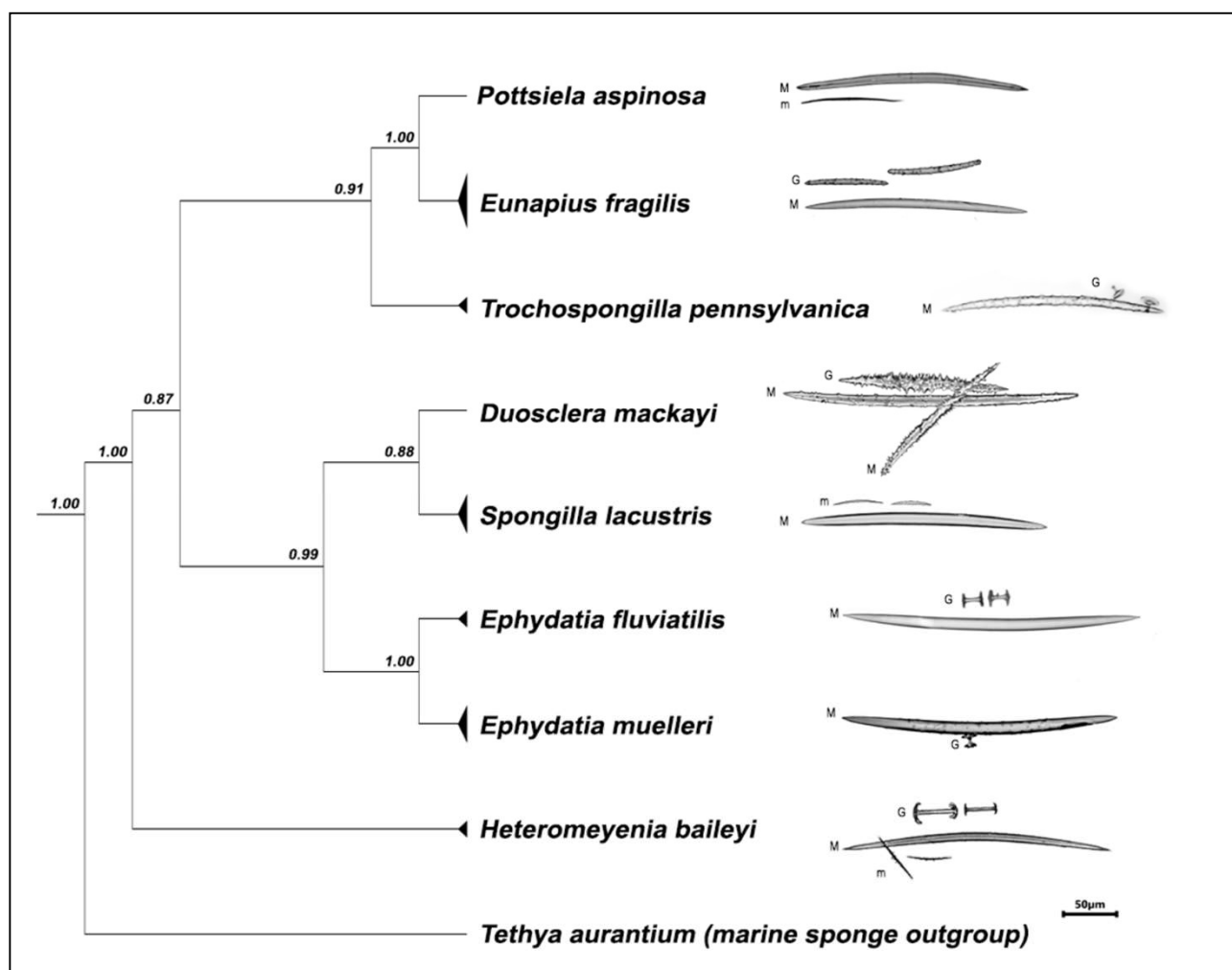**Marine demosponge selected as outgroup*

Figure 1 - Relationship of Michigan's freshwater sponge species. The summary cladogram depicts the relationships of species present in this study along with representative examples of spicules. Posterior probabilitie are indicated to the left of each node. Spicule coding: M = megasclere; m = megasclere; G = gemmulosclere.

Table S1 - Genbank accession numbers and sample location for of sequences generated during this study.

Genbank Accension	Sample ID	Species	LSSU Voucher #	Latitude	Longitude
MN856645	K02	*Eunapius fragilis*	RW0021	46.55412354	-88.21121689
MN856639	K08	*Ephydatia muelleri*	RW0023	45.70924822	-87.61000070
MT044266	K09	*Eunapius fragilis*	RW0024	45.70924822	-87.61000070
MN856640	K14	*Eunapius fragilis*	RW0025	45.70924822	-87.61000070
MN856654	K10	*Spongilla lacustris*	RW0027	45.70578486	-87.41109402
MN856638	K05	*Ephydatia muelleri*	RW0032	46.55412354	-88.21121689
MN856657	K01	*Spongilla lacustris*	RW0033	46.48178289	-89.95364405
MN856656	K13	*Spongilla lacustris*	RW0034	46.48178289	-89.95364405
MN856651	K04	*Eunapius fragilis*	RW0037	44.79399000	-83.45285000
MT044260	K16	*Eunapius fragilis*	RW0038	42.16117266	-83.40258963
MT044267	K19	*Eunapius fragilis*	RW0039	42.16117266	-83.40258963
MT044262	K34	*Eunapius fragilis*	RW0042	42.32355259	-83.83860358
MN856636	K33	*Ephydatia muelleri*	RW0043	41.87743530	-85.13791120
MN856637	K35	*Ephydatia muelleri*	RW0044	41.87743530	-85.13791120
MT044259	K15	*Eunapius fragilis*	RW0047	42.03957721	-84.26436012
MN856641	K22	*Eunapius fragilis*	RW0048	42.25626842	-85.90624150
MN856633	K20	*Ephydatia muelleri*	RW0049	46.89363300	-88.84217900
MN856659	K03	*Spongilla lacustris*	RW0050	47.46385788	-87.84262312
MN856655	K11	*Spongilla lacustris*	RW0051	46.33279200	-86.79853800
MN856661	K44	*Spongilla lacustris*	RW0052	44.43321832	-84.66962902
MN856648	K38	*Eunapius fragilis*	RW0053	44.88212400	-85.13590600
MN856631	K12	*Duosclera mackayi*	RW0054	46.60932400	-85.21435500
MT044261	K30	*Eunapius fragilis*	RW0055	43.23656803	43.23656803
MT044263	K39	*Eunapius fragilis*	RW0059	43.23656803	43.23656803
MN856646	K31	*Eunapius fragilis*	RW0056	43.23656803	43.23656803
MN856652	K48	*Heteromeyenia baileyi*	RW0063	46.20164700	-84.25398400
MN856635	K26	*Ephydatia muelleri*	RW0068	44.74710000	-84.44334000
MT111607	K17	*Pottsiella aspinosa*	RW0072	46.67527200	-85.03343000
MN856660	K40	*Spongilla lacustris*	RW0073	44.42390143	-84.67448331
MN856634	K25	*Ephydatia muelleri*	RW0074	44.74710000	-84.44334000
MN856653	K47	*Heteromeyenia baileyi*	RW0076	42.86660611	-84.40816182
MN856642	K24	*Eunapius fragilis*	RW0078	42.46615063	-84.38394025
MN856632	K37	*Ephydatia fluviatilis*	RW0080	42.67278600	-83.09614711
MN856643	K28	*Eunapius fragilis*	RW0084	42.86660611	-84.40816182
MN856650	K45	*Eunapius fragilis*	RW0085	42.46615063	-84.38394025
MN856658	K36	*Spongilla lacustris*	RW0086	44.94905100	-85.22081700
MN856662	K27	*Trochospongilla pennsylanica*	RW0087	44.94905100	-85.22081700
MN856647	K32	*Eunapius fragilis*	RW0088	42.80067612	-83.08982361
MT044265	K43	*Eunapius fragilis*	RW0089	42.68430206	-84.16596637
MT044264	K41	*Eunapius fragilis*	RW0090	42.68430206	-84.16596637
MN856649	K42	*Eunapius fragilis*	RW0099	42.46615063	-84.38394025
MN856644	K29	*Eunapius fragilis*	RW0109	42.86660611	-84.40816182
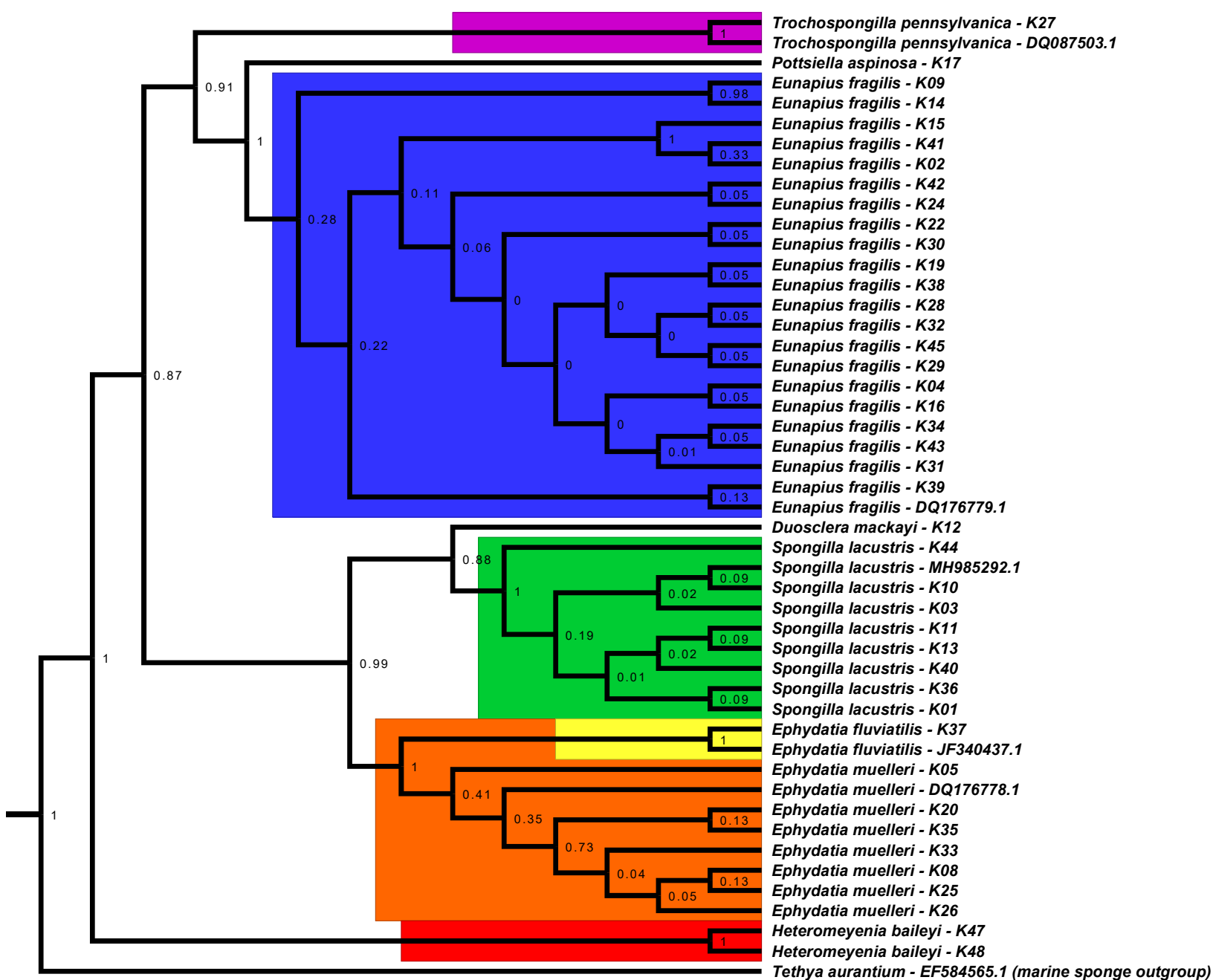
Supplemental Figure S2 - Full cladogram of all samples and reference sequences analysed during this study. Species clades are color coded for emphasis.