

Cryptic diversity and speciation in endemic *Cytherissa* (Ostracoda, Crustacea) from Lake Baikal

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## Cryptic diversity and speciation in endemic *Cytherissa* (Ostracoda, Crustacea) from Lake Baikal --Manuscript Draft--

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<b>Abstract:</b>	<p>Lake Baikal (Siberia) is the most ancient and deepest of all ancient lakes on Earth. It holds a (mostly endemic) diversity of thousands of animal species, including a speciose radiation of ostracods of the genus <i>Cytherissa</i>. Applying molecular tools to this crustacean group reveals that several morphological species are actually species clusters. Based on combined 16S and 28S DNA sequence data from thirteen classic <i>Cytherissa</i> species and one subspecies sensu Mazepova (1990), we recognize 26 different genetic <i>Cytherissa</i> species, 18 with morphological variation and eight truly cryptic species. These results suggest that the actual specific diversity of <i>Cytherissa</i> in Lake Baikal might easily be double of what is presently known.</p> <p>Baikalian endemic species most likely live in the cradle in which they originated and this opens perspectives to infer modes of speciation. Our current distribution data of <i>Cytherissa</i> species provide first indications for both geographic (lakes basins and shores) and ecological (sediment type, water depth) separation. Our present data thus provide the first steps towards future, rigorous testing of focussed hypotheses on the causality of speciation through either allopatric isolation or parapatric ecological clines.</p>	
<b>Response to Reviewers:</b>	Following the suggestions of the editor, we have adapted the parts on the ABGD and the GYMC (and omitted the references Hull 1997 and Pons 2006). We have also corrected the small errors indicated in your report. For your information, we have attached two versions of the manuscript: one with and one without track changes.	

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1 **Cryptic diversity and speciation in endemic *Cytherissa* (Ostracoda, Crustacea) from**  
2 **Lake Baikal**

3

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23

24 **Abstract**

25 Lake Baikal (Siberia) is the most ancient and deepest of all ancient lakes on Earth. It holds a  
26 (mostly endemic) diversity of thousands of animal species, including a speciose radiation of  
27 ostracods of the genus *Cytherissa*. Applying molecular tools to this crustacean group reveals  
28 that several morphological species are actually species clusters. Based on combined 16S and  
29 28S DNA sequence data from thirteen classic *Cytherissa* species and one subspecies *sensu*  
30 Mazepova (1990), we recognize 26 different genetic *Cytherissa* species, 18 with  
31 morphological variation and eight truly cryptic species. These results suggest that the actual  
32 specific diversity of *Cytherissa* in Lake Baikal might easily be double of what is presently  
33 known.

34 Baikalian endemic species most likely live in the cradle in which they originated and this  
35 opens perspectives to infer modes of speciation. Our current distribution data of *Cytherissa*  
36 species provide first indications for both geographic (lakes basins and shores) and ecological  
37 (sediment type, water depth) separation. Our present data thus provide the first steps towards  
38 future, rigorous testing of focussed hypotheses on the causality of speciation through either  
39 allopatric isolation or parapatric ecological clines.

40 **Keywords:** allopatric speciation, parapatric speciation, depth distribution, sediment types,  
41 lake basins, east-west shores, sexual reproduction

42

43

## 44 **Introduction**

45 Ancient lakes are natural laboratories for evolutionary studies on the tempo and mode of  
46 speciation of their endemic fauna and flora (Martens, 1997). There are only a couple of  
47 dozens of these lakes on the globe and they are hotspots of biodiversity, because of their high  
48 endemism and their importance for generating diversity in surrounding areas (Schön &  
49 Martens, 2012). Non-marine ostracods are not only an important ecological component of  
50 ancient lake taxa, but ancient lakes also contribute up to 25% of all known non-marine  
51 specific ostracod diversity (Martens et al., 2008).

52 On a global scale, Lake Baikal is the oldest extant lake with an estimated age of 25-30 myr  
53 (Sherbakov, 1999; Müller et al., 2001) as well as the deepest lake with a maximal depth of  
54 more than 1600 m (Sherstyankin et al., 2006). About 2500 animal (morpho) species occur in  
55 Lake Baikal, of which 1455 are endemic (Timoshkin, 2001). Concerning non-marine  
56 Ostracoda, more than 90% of Baikalian ostracods are endemic to the lake, and the *Cytherissa*  
57 species flock has the highest specific diversity (Mazepova, 1990). This species flock is  
58 probably an example of explosive radiation and has originated 5-8 million years ago (Schön  
59 & Martens, 2012), at a time when Lake Baikal's cold, oxygenated abyss was formed  
60 (Sherbakov, 1999).

61 With the availability of molecular tools, the last twenty years have seen an ever-increasing  
62 number of studies detecting so-called cryptic diversity (Bickford et al., 2007), i.e. lineages  
63 that are morphologically similar but fulfil all criteria to be different genetic species (Vogler &  
64 Monaghan, 2007) according to the phylogenetic species concept (Eldredge & Cracraft  
65 (1980), but see also overview in Zhang et al., 2013) or the evolutionary genetic species  
66 concept (Birky & Barraclough, 2009). There is mounting evidence that cryptic species occur  
67 widely and that their presence is, at least in part, linked to specific types of habitat. For

68 example, freshwater taxa show significantly more cryptic diversity than either terrestrial or  
69 marine taxa (Poulin & Pérez-Ponce de León, 2017).

70 Also in non-marine ostracods, cryptic species have been detected, varying between eight in a  
71 putative ancient asexual darwinulid species (Schön et al., 2012) to more than 35 in a single  
72 Holarctic temporary pool species (Bode et al., 2010). Likewise, cryptic species have been  
73 found in endemic *Romecytheridea* ostracods from Lake Tanganyika, the second most ancient  
74 lake in the world (Schön et al., 2014). The discovery of cryptic lineages throughout all  
75 metazoan phyla (Beheregaray & Cacccone, 2007; Pfenninger & Schwenk, 2007) is not only  
76 important for fundamental science and systematics, but has also profound implications for  
77 conservation and management (examples in Brown et al., 2007; Elmer et al., 2007; Fontaneto  
78 et al., 2008; Gustafsson et al., 2009; Marrone et al., 2010), especially in unique environments  
79 such as ancient lakes. Indeed, if genetic diversity is cryptic, it is equally difficult to recognize  
80 it and to protect it from extinction.

81 Here, we use mitochondrial and nuclear DNA sequence data to test for the presence of cryptic  
82 species within 14 known morphological *Cytherissa* species and subspecies. Our samples  
83 come from all three basins of Lake Baikal, from both eastern and western shores and from  
84 both different water depths and different types of sediments, enabling us to assess the recent  
85 distribution patterns of these ostracods. Our research provides preliminary indications on the  
86 causal importance of allopatric isolation (different basins or shores) and parapatric ecological  
87 speciation (depth, sediment types) for the past radiation of endemic *Cytherissa* species in  
88 Lake Baikal.

89

90 **Material and Methods**

91 *Sampling*

92 During four expeditions on Lake Baikal, in 1999, 2007, 2009 and 2011, several dozens of  
93 samples for ostracods were collected by SCUBA diving, trawling, dredging and with the  
94 oceanographic Reineck box-corer, from various locations in Lake Baikal, including both the  
95 eastern and western shore and all three basins and at depths as from 20m in the littoral photic  
96 zone (0–100 m) to deep water habitats of more than 500 m. Ostracods were sorted alive under  
97 a light microscope on the research vessels, were fixed in cold 95% pure ethanol for  
98 subsequent analyses and separated into preliminary taxonomic groups using the valve  
99 outlines of Mazepova (1990) and the hemipenis outlines by Van Mulken et al. (in prep.). We  
100 also sampled *Cytherissa lacustris*, the recent extra-lacustrine spin-off of the Baikalian  
101 *Cytherissa* flock (Schön & Martens, 2012), from Semerwater in the UK (see Table 1 for more  
102 details).

103

104 *DNA extraction, PCR and sequencing*

105 For most specimens, valves were removed for Scanning Electron Microscopy (SEM) and the  
106 remaining soft parts were used to extract DNA from individual ostracods with a slightly  
107 modified protocol of the DNA Easy Blood and Tissue kit (Qiagen), adjusting the elution  
108 volumes because of the small size of individual ostracods. We estimated the concentration of  
109 the obtained DNA extractions with the Nanodrop and used the eluate with the highest  
110 concentration for all subsequent steps of the molecular analysis. With PCR (Polymerase  
111 Chain Reaction), we amplified part of the mitochondrial 16S ribosomal region with specific  
112 primers (16S-F3 TTAATTCAACATCGAGGTCACAA and 16S-R2  
113 GAGTAAACGGCTGCAGTA) and the D1D2 part of the nuclear Large Subunit (28S) with  
114 universal primers (D1D2Fw1 5'-AGCGGAGGAAAAGAACTA-3') and (D1D2Rev1 5'-

115 TACTAGAAGGTTTCGATTAGTC-3') (Sonnenberg et al., 2007). Both regions have been  
116 successfully sequenced in other studies on non-marine ostracods (Bode et al., 2010; Koenders  
117 et al., 2012; Schön et al., 2014), and have also been used for the detection of cryptic species.  
118 PCRs were conducted in a T personal Thermoblock (Biometra) with 25 µl volumes of the  
119 Qiagen HotStar Mastermix (1.5 mM MgCl<sub>2</sub>, 200µM dNTP, Tris·Cl, KCl, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.25 U  
120 Taq), 0.1 µM of each primer and the following cycling conditions: 15 min at 95°C, followed  
121 by 40-42 cycles of 1 min at 95°C, 1min at 44°C (16S) and 48°C (28S) and 1 min at 72°C,  
122 followed by a final extension step of 72°C for 10 minutes. We used agarose gel  
123 electrophoresis and stained gels with GelRed to check if PCR amplifications were successful.  
124 Positive amplicons were purified with the GFX PCR DNA and gel band purification kit (GE  
125 Healthcare) kit and sequenced in both directions using the PCR primers and the Big Dye kit  
126 (ABI) on an ABI 3130x1 capillary DNA sequencer (Life Technologies).

127

#### 128 *Analyses of DNA sequence data*

129 We visualized sequencing chromatograms and generated consensus sequences for each  
130 specimen with Bioedit (Hall, 1999). Sequence ambiguities were checked by eye and  
131 corrected manually, sequences were aligned with MAFFT (Katoh & Standley, 2013) on  
132 <http://www.ebi.ac.uk> and trimmed to equal lengths in BioEdit. Sequence identity was  
133 confirmed by BLAST searches in Genbank (Altschul et al., 1997). As outgroup, we used  
134 sequence data from *Romecytheridea ampla*, an ostracod species from Lake Tanganyika  
135 belonging to the same subfamily and from which both 16S and 28S sequence data were  
136 available (Table 1). We also combined the DNA sequence data from both markers into a  
137 congruent dataset with Sequence Matrix (Vaidya et al., 2011). We trimmed the final  
138 alignment for each dataset with the outgroup (Table 1) to equal length and selected the best-



139 fitting evolutionary model in jModeltest 2 (Darriba et al., 2012) using model filtering, the  
140 corrected Akaike Information Criterion (AICc) and 88 different nucleotide substitution  
141 models. The parameters of the best-fitting evolutionary models were used in phylogenetic  
142 reconstructions with Maximum Likelihood (ML) (PHYML; Guindon & Gascuel, 2003) and  
143 Bayesian approaches (MrBayes 3.2; Ronquist et al., 2012). Not all models selected by  
144 jmodeltest2 are implemented in MrBayes and we therefore had to pick the closest ones for  
145 Bayesian analyses. For 16S, the TIM1+I+G model was selected with freqA = 0.2927; freqC =  
146 0.2296; freqG = 0.1968; freqT = 0.2810; [AC] = 1.0000; [AG] = 5.4559; [AT] = 0.6273;  
147 [CG] = 0.6273; [CT] = 3.1987; [GT] = 1.0000; p-inv = 0.2280; gamma shape = 0.4750.  
148 For 28S, the TPM3uf+ G model was selected with the following parameters: freqA = 0.1981;  
149 freqC = 0.2286; freqG = 0.3059; freqT = 0.2675; [AC] = 0.0000; [AG] = 3.6775; [AT] =  
150 1.0000; [CG] = 0.0000; [CT] = 3.6775; [GT] = 1.0000; gamma shape = 0.3010. For the  
151 combined dataset, the TIM1+I+G model was selected with freqA = 0.2632; freqC = 0.2145;  
152 freqG = 0.2316; freqT = 0.2907; [AC] = 1.0000; [AG] = 5.3221; [AT] = 0.5999; [CG] =  
153 0.5999; [CT] = 3.5948; [GT] = 1.0000; p-inv = 0.4740; gamma shape = 0.3080. In all cases,  
154 we constructed ML trees in PHYML with these parameters and 1000 bootstraps. We also  
155 constructed ML trees without bootstrap support and outgroups and from haplotype  
156 sequencing only for the Poisson Tree Processes (PTP) algorithm (Zhang et al., 2013) to  
157 delimitate genetic species (see below). For Bayesian approaches, we ran MrBayes with two  
158 MCMC chains and 20 million generations, applying the GTR+I+G model for 16S and the  
159 combined dataset and the HKY85+G model for 28S, and sampling trees every 1000  
160 generations. After inspecting the results, we eliminated the first 20000 trees as burn-in and  
161 calculated the 50% majority rule consensus tree. All trees were visualized and manipulated  
162 with MEGA 6.0 (Tamura et al., 2013) and FigTree (Rambaut, 2017).

163

164 *Networks*

165 To obtain the best graphic representation of haplotypes and their connectivity at the  
166 population level, we also constructed minimum spanning (Bandelt et al., 1999) networks  
167 from the 16S and 28S data with popart 1.2 (<http://popart.otago.ac.nz>) colour-coding the  
168 geographic origin (lake basin) as traits.

169

170 *Delimitating genetic species*

171 We used two different methods for quantitative delimitations of genetic species based on the  
172 evolutionary genetic species concept (Birky & Barraclough, 2009), nl. the 4  $\theta$  (theta) rule  
173 (Birky et al., 2010; Birky, 2013) and the PTP algorithm (Zhang et al., 2013). For applying the  
174 4  $\theta$  rule, we first identified well-supported phylogenetic sister clades from the ML and  
175 Bayesian phylogenies with a bootstrap support of more than 75% or a posterior probability of  
176 more than 0.8. Within and between the sister clades, we then calculated genetic distances in  
177 MEGA 6.0 using the appropriate model for molecular evolution. As with Bayesian analyses,  
178 not all models selected by jmodeltest2 are available in MEGA and we chose the closest ones  
179 for the calculation of genetic distances. Next,  $\pi$  (nucleotide diversity) and  $\theta$  (population  
180 mutation rate) were calculated taking sampling size of each sister clade into account. Finally,  
181 we calculated D (distance between sister clades) and the ratio between  $\theta$  and D. If the  
182 resulting ratio is greater than 4, sister clades are considered to be different evolutionary  
183 species (Birky et al., 2010).

184 We also used a Poisson Tree Processes (PTP) model to delimit genetic species. This  
185 algorithm is based on a shift of the Poisson distributions of substitution rates of branches  
186 within and between species in a phylogenetic tree (Zhang et al., 2013) The ML trees of 16S,  
187 28S and the combined 16S/28S dataset were uploaded on the website of bPTP (<http://sco.h-its.org/exelixis/web/software/PTP>) without outgroups and bootstraps and only representing

189 individual haplotypes . The statistical support of potential genetic species was calculated with  
190 the maximal possible number of 500,000 MCM generations and the default burn-in of 10%.  
191 For comparisons, we also applied a third approach for genetic species delimitations, the  
192 Automatic Barcode Gap Discovery method (ABGD; Puillandre et al., 2012) which calculates  
193 genetic distances between all sequences and does not require any phylogenetic information.

194

#### 195 *Statistical analyses of current distribution data*

196 We summarize current distribution data of all genetic species defined by the congruent  
197 molecular data sets regarding ecological (sediment type, water depth), and geographic  
198 (different lake basins, different shores) factors. We also compare our ecological distribution  
199 data to the much larger dataset of Mazepova (1998) on different sediment types and water  
200 depths of morphological *Cytherissa* species and subspecies.

201 We then generated a presence-absence matrix for each genetic species from the combined  
202 molecular dataset for the four distribution variables, using our geographic and ecological data  
203 and the ecological data of Mazepova (1998). This data matrix was used for ordination  
204 analyses in PAST (Hammer et al., 2001). More specifically, we conducted a Principal  
205 Coordinate Analysis (PCoA) with the jaccard similarity index, and the default transformation  
206 exponent of 2. This kind of analyses plots the distribution of genetic *Cytherissa* species in a  
207 coordination system where the axes are linked to the different distribution variables.

208

## 209 **Results**

210

### 211 *DNA extraction*

212 We have extracted DNA from more than 100 specimens, and have been successful in  
213 obtaining 68 sequences for 16S and 83 sequences of 28S, respectively (Table 1). Developing

214 suitable primers for 16S has been a major obstacle and has involved several rounds of  
215 redesigning both forward and reverse primers. Problems with the primers are also the reason  
216 why we could not successfully follow the approach of Schön & Martens (2012) in acquiring  
217 more COI sequences from the same species and localities, which would have been very  
218 useful for further comparisons. Also, the specimens or DNA extractions of Schön & Martens  
219 (2012) were no longer available to be included in the current study.

220

## 221 *Molecular taxonomy*

### 222 *Combined molecular datasets*

223 Combining both molecular datasets has resulted in phylogenetic trees with some higher  
224 support for deeper nodes in the upper part of the tree (Figure 2) than the phylogenies that  
225 were based only on 16S (Figure S1) or 28S (Figure S2). The terminal branches in Figure 2  
226 are generally well supported with bootstrap values of 75% or more and posterior probabilities  
227 of more than 0.8. In the combined 16S/28S tree, such well-supported clades consist of sister  
228 groups (*C. lacustris* I and II; *C. golyschkiniae* I and II; *C. parallela* I and II) but also of  
229 clusters of different morphological (sub)species (*C. parva* and *C. sp. 3*; *C. parallela* III and  
230 both *C. lacustris* I and II; *C. sernovi insularis* I and *C. sernovi sernovi*). The remaining part  
231 of the tree, however, still contains many polytomies, especially at the deeper nodes. With the  
232 exception of *C. lata* I and II and *C. tuberculata tuberculata* IV and V, respectively, the  
233 phylogenetic relationships of the eight other clades remains unresolved.

234 The 16S and 28S DNA sequences come from 13 known morphological species and one  
235 subspecies *sensu* Mazepova (1990) plus four new species that await formal description  
236 elsewhere. With the combined molecular data, we identified 26 well-supported phylogenetic

237 clades (Figure 2). Many of these are congruent with morphological species (*C. parva*, *C.*  
238 *pterygota*, *C. interposita*, *C. excelsiformis*, *C. glomerata* and four yet undescribed species (*C.*  
239 *spec.* 1 to 4) plus one subspecies (*C. sernovi sernovi*). There are an additional five  
240 morphospecies with multiple, well-supported phylogenetic clades or with phylogenetically  
241 distant sister clades, both indicating possible cryptic species. *Cytherissa tuberculata*  
242 *tuberculata* splits into four such clades and *C. parallela* into three, while two each are found  
243 in *C. lacustris*, *C. sernovi insularis*, *C. golyschkiniae*, *C. sinistrodentata*, and *C. lata* (Figure  
244 2).

245 We have used two different methods to test if these phylogenetic clades fulfil the criteria to  
246 be considered different evolutionary genetic species. Because of the more limited number of  
247 specimens for which DNA sequence data are available from both genomic regions, the  
248 number of singletons in the congruent phylogeny (Figure 2) is larger than in the 16S tree  
249 (Figure S1). Singletons cause potential problems when applying the 4  $\theta$  rule (see below).  
250 Still, this method supports 17 genetic species within morphospecies (Table 2, Table S1) plus  
251 another eight morphospecies (Figure 2). The PTP algorithm recognizes all of the clades from  
252 the 4  $\theta$  rule (Table 2) and additionally splits *C. parallela* I and II into two different genetic  
253 species with one singleton each (Table 2). The ABDG method delimitates the same species as  
254 PTP (when using the 16S data for ABDG; not shown, data are available from IS on request).  
255 We take a conservative approach in delimitating species, using support from all three  
256 methods and therefore regard the two clades of *C. parallela* I and II for now as two genetic  
257 species. We can then recognize a total of 26 different genetic species from the combined  
258 molecular data. For most of these genetic species, we find variation in valve characters  
259 (indicated in bold in Figure 2), thus providing morphological support for genetic species  
260 boundaries. Many of these species are also the same as in Mazepova (1990). We also found  
261 four new morphological species (*C. sp.* 1 to 4) that are, with the exception of *C. sp.* 1, also

262 fully supported by the combined molecular data. Other genetic species resemble the species  
263 *sensu* Mazepova (1990) to some extent but show additional valve differences. These species  
264 are still awaiting a formal taxonomic description and are for now indicated with Roman  
265 numbers after the original species name in Figure 2 (*C. parallela* I-III and *C. tuberculata*  
266 *tuberculata* II-V - see Fig S3, SEM plate). However, in the other instances of genetic species  
267 with Roman numbers in Figure 2, no clear morphological differences are found and these  
268 eight remaining lineages are here considered to be true cryptic species.

269

#### 270 *16S results*

271 Because we could obtain more sequence data from this marker than could be used for the  
272 combined data set, numbers of genetic species are slightly different. If we apply the PTP  
273 algorithm or the ABDG method (not shown, data are available from IS on request), we can  
274 identify 35 genetic species (Figure S1). With the 4  $\theta$  rule, the result is, with 36 genetic  
275 species, rather similar, but individual species delimitations are incongruent for morphospecies  
276 with several genetic species (Table S3).

277 When comparing the 16S species boundaries to morphological variability as we did for the  
278 combined molecular data set, we find a total of nine truly cryptic species in the 16S dataset  
279 (Figure S1), one more than with the 16S/28S data.

280 The structure of the 16S minimum spanning network (Figure S4) matches the well-supported  
281 phylogenetic clades in Figure S1. We find 58 different haplotypes that are separated from  
282 each other by more than 20 mutational steps. Within evolutionary genetic species such as for  
283 example *C. tuberculata tuberculata* III, *C. lacustris* II or *C. golyschkiniae* IV, we also find  
284 haplotypes differing only by small numbers of mutational steps.

285

286 *28S results*

287 The nuclear ribosomal 28S region shows very little genetic variability amongst Baikalian  
288 *Cytherissa* species. Consequently, the phylogenetic tree is unresolved with very few  
289 exceptions (see Figure S2). There are only 18 haplotypes in the minimum spanning network,  
290 with a maximum of four mutation steps (Figure S5) although more than 80 specimens from  
291 have been sequenced from the entire lake. The network shows three very common 28S  
292 haplotypes (Figure S5). The most frequent one is present in more than ten different  
293 morphospecies and subspecies. Except for one specimen of *C. golyschkiniae* and *C. verrucosa*  
294 each that are separated by four mutation steps from the next haplotype (Figure S5), all other  
295 single 28S haplotypes are only one or two mutational steps away from the three most  
296 common haplotypes or from each other. Because of the limited genetic diversity of 28S, we  
297 did not use these DNA sequence data to delimitate genetic species boundaries.

298

299 *Current distribution of genetic Cytherissa species*

300 Our sampling scheme contains habitats with different ecological (sediment type, water depth)  
301 and geographic features (south, central and northern basin; and east and west shores), which  
302 could have contributed to different distributions of the genetic *Cytherissa* species. Because  
303 our sample numbers are somewhat limited, we have compared our distribution data to the  
304 larger dataset of Mazepova (1998; Table S2). It seems that certain morphological  
305 (sub)species have previously been found on more sediment types than in our study (e. g. *C.*  
306 *golyschkiniae*, *C. tuberculata tuberculata*, *C. excelsiformis* and *C. glomerata*; Table S2).  
307 Mazepova (1998) also reported a wider depth distribution for these three morphological

308 (sub)species as well as for *C. sinistrodentata*. For the remaining seven genetic species, our  
309 data match the depth distributions of Mazepova (1998) well.

310 Table 3 summarize the distribution data of all genetic species from the congruent molecular  
311 dataset, arranged in pairs of genetic sister clades to allow easy comparisons. A PCoA analysis  
312 of these data shows that most genetic species are well separated from each other (Figure 3),  
313 also the species pairs from Table 3 and the various cryptic species (see above). The first axis  
314 with an eigenvalue of 1.5139 explains 35.6% of the overall variation and the second one  
315 22.03 %, which are relatively high scores.

316



317 **Discussion**

318 *Phylogenetic and network structures*

319 We have sequenced two different genetic markers, namely part of the mitochondrial 16S and  
320 part of the nuclear 28S ribosomal region from 18 morphological (sub)species of *Cytherissa*.  
321 The molecular phylogenies from both genomic regions show many polytomies, especially of  
322 the deeper nodes, regardless of the methods used for phylogenetic reconstructions (Figure 2,  
323 S1 & S2) and when using the two datasets either separately or combined (Figures 2, S1-S2).  
324 In our 16S and combined 16S/28S trees, only the terminal nodes and some deeper nodes  
325 (16S/28S) are statistically well supported (Figure 2) whereas almost the entire 28S phylogeny  
326 remains unresolved (Figure S2). Our phylogenetic results thus resemble those of Schön &  
327 Martens (2012), as also in their study, the mitochondrial phylogeny of Baikalian *Cytherissa*  
328 ostracods based on COI had many polytomies, and only the terminal nodes were well  
329 supported in the mitochondrial gene (COI), while the nuclear phylogeny (from the ITS1  
330 region) was not resolved at all. Similar incongruences in genetic variability between  
331 mitochondrial and nuclear markers have also been reported from other studies on ostracods  
332 (Schön et al., 1998, 2010, 2012, 2014; Brandao et al., 2010; Koenders et al., 2012), and on  
333 meiofauna in general (Tang et al., 2012), resulting in low phylogenetic resolution and  
334 polytomies. One potential causality for this discrepancy is that nuclear ribosomal regions in  
335 non-marine ostracods generally seem to evolve at a much slower pace than mitochondrial  
336 regions (Schön et al., 2003). Even more relevant here is the detection of explosive speciation  
337 in Baikalian *Cytherissa* (Schön & Martens, 2012), which explains best why our phylogenies  
338 are unresolved at the base of the trees.

339 The topology of our combined tree (Figure 2) reveals certain similarities with the trees in  
340 Schön and Martens (2012), as *C. tuberculata* is closest to the root of the *Cytherissa* flock and

341 *C. parva* forms a well-supported clade with *C. sp. 3*, apart from the other *Cytherissa* species.  
342 However, in our present results we cannot detect the four well-supported clades from the COI  
343 tree of Schön & Martens (2012). These inconsistencies could be owing to differences in  
344 genetic variability between 16S and COI and the fact that our dataset is not fully congruent  
345 with the data of Schön & Martens (2012). Because these authors used other specimens, it is  
346 also not possible to combine and re-analyse all existing molecular data of *Cytherissa*. The  
347 problem of resolving the Baikalian *Cytherissa* phylogenies urgently calls for the development  
348 of more suitable, large scale molecular markers such as sequencing entire mitogenomes  
349 (Schön & Martens, 2016) or large scale genomic data from multiple markers, such as those  
350 Meyer et al. (2015) developed for cichlid fish.

351

### 352 ***Diversity of Baikalian Cytherissa***

353 Our results show that the actual biodiversity of endemic *Cytherissa* in Lake Baikal is higher  
354 than previously thought. Mazepova (1990) recognized a total of 47 species and 10 subspecies  
355 of *Cytherissa* based on valve characters. We have used SEM (see above and Fig S3) to study  
356 differentiation of valve morphology in all specimens and also characterised hemipenis  
357 morphology for selected *Cytherissa* species (Van Mulken et al. in prep.). Both methods  
358 provide a much finer resolution of morphological differentiation, as is illustrated by the four  
359 (16S/28S combined) to five (16S) new *Cytherissa* species that we found and that are  
360 confirmed with our genetic data. Also, in three *Cytherissa* morphospecies *sensu* Mazepova  
361 (1990), we can distinguish nine genetic *Cytherissa* species with clear differences in valve  
362 morphologies, that also fulfil the criteria of the evolutionary genetic species concept using the  
363 combined DNA sequence data (*C. parallela* I-III, *C. lacustris* I & II, *C. tuberculata*  
364 *tuberculata* II-V; see Figure S3 for the latter).

365 We have furthermore detected several truly cryptic species (without any apparent  
366 morphological differentiation), which supports the first indications reported by Schön &  
367 Martens (2012) for cryptic speciation in Baikalian ostracods. Using the combined molecular  
368 data set, we identify eight cryptic species (nine when only using the 16S; see Figure S1).  
369 When applying two different statistical methods to delimitate evolutionary genetic species,  
370 the 4  $\theta$  rule and the PTP algorithm, the overall estimate was for the combined dataset  
371 relatively similar with 26 and 27 species, respectively.

372 But our combined 16S/28S tree has many well supported clades with singletons (Figure 2),  
373 and the genetic diversity within such clades is zero. Consequently, the ratios would have to  
374 be divided by zero and can thus not be calculated (Table S1). The results of the PTP  
375 algorithm are probably more robust as this method can also be used for singletons and we  
376 found furthermore the same genetic species when applying the ABDG method and this for  
377 both 16S and the combined molecular dataset. We could not increase the number of  
378 specimens in our study, because of the difficulties to obtain more 16S sequences (see above)  
379 and because of low densities of non-marine ostracods in Lake Baikal, especially at greater  
380 depths.

381 In total, we can identify 26 different genetic species with the combined molecular data set,  
382 representing 14 morphological (sub)species *sensu* Mazepova (1990). Our data thus almost  
383 double the previously known diversity of endemic *Cytherissa* species from Lake Baikal. Our  
384 sampling includes all three basins of Lake Baikal, five different sediment types and water  
385 depths ranging from shallow habitats (c 20 m) to more than 500m, covering most of the  
386 habitat and geographical diversity of Lake Baikal. Extrapolating our results on morphological  
387 and cryptic diversity to the entire Baikalian *Cytherissa* species flock (26 genetic species in 14  
388 morphospecies *sensu* Mazepova (1990)) implies that we can expect almost twice as many  
389 (cryptic) *Cytherissa* species from Lake Baikal as previously known, with therefore one

390 hundred species, including cryptic ones, being a more realistic estimate than the 47  
391 morphological (sub)species *sensu* Mazepova (1990) previously known. Studies on ostracods  
392 from other ancient lakes have also reported the presence of cryptic species, thus considerably  
393 increasing classic diversity estimates (Schön et al., 2014; Karanovic, 2015). Likewise, cryptic  
394 species have also been found in Baikalian amphipods (Vainola & Kamaltynov, 1999) and in  
395 Baikalian sponges (Itskovich et al., 2015).

396 High cryptic diversity and cryptic speciation in ancient lakes somewhat negates the recent  
397 findings by Poulin & Pérez-Ponce de León (2017), who attributed the higher cryptic diversity  
398 in freshwater as compared to terrestrial and marine habitats, to the greater heterogeneity of  
399 freshwater habitats. This hypothesis mostly refers to the patchiness and isolation of the many  
400 freshwater pools, lakes and rivers. However, in the case of ancient lakes, their long  
401 evolutionary history, large size and unusual depth are probably more important for generating  
402 cryptic diversity than providing many heterogeneous habitats.

403 Based on classic morphological species boundaries, ancient lakes have already been  
404 identified as major hotspots for non-marine ostracod diversity as, for example, they  
405 contribute 25% of all known freshwater ostracod species (Martens et al., 2008). The increase  
406 of the known diversity through the discovery of cryptic species from our and other studies  
407 emphasises the importance of ancient lakes as biodiversity hot spots, not only for ostracods.  
408 This has major implications for the conservation and protection of these lakes and their  
409 unique fauna and flora, even outside the lakes themselves (Schön et al., 2000; Schön &  
410 Martens, 2012)

411

412 ***Factors linked to speciation in Baikalian Cytherissa***

413 Ancient lakes are *in situ* laboratories for evolutionary studies in general, and to investigate  
414 the factors that have promoted and caused speciation, giving rise to the impressive endemic  
415 diversity of these lakes in particular. Mayr (1942, 1963) regarded geographic isolation as the  
416 most important driver for (allopatric) speciation and this view dominated the field for a long  
417 time. Meanwhile, also the importance of intrinsic factors for sympatric speciation in ancient  
418 lakes has been recognized (see, for example, Schön & Martens, 2004 and Cristescu et al.,  
419 2010), with cichlid fish still being the most prominent example (e. g. Muschick et al., 2012).  
420 Martens (1994, 1997) furthermore re-iterated the term “parapatric speciation”, describing  
421 isolation and gene flow along an ecological or geographical gradient, which is highly  
422 applicable to Lake Baikal with its deep, fully oxygenated abyss (down to 1600 m), and its  
423 north-south length of more than 600 km (Martin, 1994). Because our study detected at least  
424 26 genetic species of *Cytherissa*, including some cryptic species, and because our sample  
425 scheme included all three basins of Lake Baikal from the eastern and western shore, depths  
426 ranging from shallow to deep water habitats and five sediment types, we can make a first  
427 attempt to assess how recent *Cytherissa* species could be ecologically and geographically  
428 separated. Because of the limited number of molecular data currently available and the lack  
429 of extensive, dated phylogenies, our analyses can only provide the very first steps towards  
430 future, rigorous testing of hypotheses on allo- or parapatric speciation of non-marine  
431 ostracods in Lake Baikal in general and of selected *Cytherissa* clades in particular.

432

### 433 *Geographic and ecological separation*

434 Geographic separation because of historical vicariance might to some extent have shaped  
435 *Cytherissa* diversity, and possibly, also speciation in Lake Baikal. Our PCoA illustrates that  
436 most genetic *Cytherissa* species are clearly separated by extrinsic factors (Figure 3), even if  
437 we use the (wider) ecological distribution data of Mazepova (1998) for water depths and

438 sediment for morphological species and subspecies without being able to differentiate further  
439 according to our genetic species (see Table S2). What is more difficult to assess is the extent  
440 to which each factor might have contributed to the current ecological and geographic  
441 distribution and to speciation in the past. We find several examples where different (cryptic)  
442 *Cytherissa* species seem to be limited in their geographic distribution to a single Baikalian  
443 basin or shore (species pairs: *C. lacustris* I & II; *Cytherissa sernovi insularis* II & *C. sernovi*  
444 *sernovi*, *C. lata* I & II, *C. tuberculata tuberculata* IV & V; see Table 3). Distribution patterns  
445 potentially resulting from allopatric speciation amongst basins have also been described from  
446 Lake Tanganyika for ostracods (Schön et al., 2014) and cichlid fish (Snoeks et al., 1994;  
447 Rüber et al., 1999, 2001; Sturmbauer et al., 2001; Nevado et al., 2009, 2011). Keeping the  
448 lack of ancestral reconstructions and thus rigorous testing of this hypothesis in mind, our  
449 preliminary indications for allopatric speciation in Lake Baikal are still noteworthy as only  
450 one other case of supposed allopatric speciation from this lake is documented up to now,  
451 namely the case of *Eulimnogammarus cyaneus* versus *E. messerschmidtii* (Bedulina et al.,  
452 2014).

453 Other examples in our dataset show that besides geographic separation, also ecological  
454 factors like water depths or sediment types might have further contributed to the current  
455 disjunct distribution of certain *Cytherissa* species (e.g. the species pairs *C. parallela* I & II;  
456 *C. lata* I & II and *Cytherissa tuberculata tuberculata* IV & V, see Table 3).

457 For most of the genetic *Cytherissa* species, our depth ranges match the ones of Mazepova  
458 (1998) remarkably well. Exceptions include *C. glomerata* but also the common  
459 morphospecies *C. sernovi*, *C. sinistrodentata* and *C. tuberculata tuberculata*, for which  
460 Mazepova (1998) reported much wider depth distributions than we found for our different  
461 genetic species constituting these classic morphospecies (Table S2). Whether or not there is  
462 indeed a clear separation between the genetic/ cryptic species by water depths and/or by

463 sediment type (as in snails from Lake Tanganyika; Michel et al., 1992) still has to be further  
464 tested with more extensive sampling and subsequent genetic characterisation.

465

#### 466 *Potential intrinsic factors*

467 We can currently not assess at all to which extent intrinsic factors or adaptive evolution have  
468 caused sympatric ostracod speciation. To investigate trophic niches, for example, detailed  
469 morphological investigations of the appendages involved in food processing or stable isotope  
470 analyses would be needed. Preliminary analyses of soft part morphology in *Cytherissa* offers  
471 no indication for trophic specialisation in the relevant head appendages (Danielopol & Tétart,  
472 1990).

473 To study other intrinsic mechanisms, like for example hybridisation or introgression which  
474 are common in cichlids from African ancient lakes (Koblmüller et al., 2007; Nevado et al.,  
475 2009, 2011; Cristescu et al., 2010; Genner & Turner, 2011; Anseeuw et al., 2012; Meier et  
476 al., 2017), is not possible to date as no suitable molecular tools are as yet available for  
477 ostracods. It remains therefore uncertain whether the large number of 28S haplotypes shared  
478 between different *Cytherissa* species (Figure S5) is a first indication for hybridisation or is  
479 merely a reflection of the low variability of this nuclear region.

480 Sexual selection has often been cited as a major driver in ancient lake speciation, and the best  
481 documented examples are of course (again) the cichlid fish (reviewed in Wagner et al. 2012).

482 Sexual selection in ostracods has previously been documented in both freshwater (Martens,  
483 2000) and marine groups (Tsukagoshi, 1988), and is often detectable by wide morphological  
484 differences in copulatory structures (hemipenes, prehensile palps) between otherwise closely  
485 related species. However, the study by Van Mulken et al. (in prep.) shows that the copulatory  
486 appendages in Baikalian *Cytherissa* species, albeit quite elaborate, are very similar amongst  
487 otherwise different species.

488 *Cytherissa lacustris* II is found in both Lake Baikal and in the UK (Table 1; Figure S4; Schön  
489 & Martens, 2012) and has different reproductive modes. In Lake Baikal, its “sors” (shallow  
490 lagunas associated with the lake) and in Lake Huvsugul (Mazepova, 2006) it is fully sexual,  
491 while in the rest of the Holarctic it is obligate asexual (Schön et al., 2000; Schön & Martens,  
492 2012). This variation in reproductive mode indicates that such intrinsic factors might also be  
493 relevant for ostracod speciation in ancient lakes (Martens, 1994) and elsewhere, and need to  
494 be studied with suitable tools.

495

## 496 **Conclusions**

497 To summarize, we found strong evidence from two molecular markers and morphological  
498 variation that the *Cytherissa* diversity from Lake Baikal is probably twice as large as  
499 previously known. Our preliminary data also indicate that the 26 genetic species are to some  
500 extent separated by ecological (sediment types, water depths) as well as geographic (basin,  
501 shore) factors. We argue that these separations might have been causal to allopatric and  
502 parapatric speciation along gradients without complete isolation. In the case of Lake Baikal,  
503 such gradients include the vast geographic distances among the three basins, between the two  
504 shores (east and west) and the large ecological gradient in water depth down to c 1600 m.  
505 These hypotheses will need to be rigorously tested in future research.

506 Other external factors possibly promoting speciation, such as multiple invasions, have  
507 already been documented (Schön & Martens, 2012), while also adaptive and intrinsic  
508 components are expected to have further contributed to generating the high diversity of  
509 Baikalian ostracods and other endemic taxa. We hope that with the increasing availability of  
510 various “omics” techniques, also applicable to ostracods (Schön & Martens, 2016), future  
511 studies will be able to answer these fundamental questions of evolutionary biology, and in  
512 particular on speciation in ancient lakes.



513

514

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734

735

736 **Captions of Figures and Tables**

737 **Tables:**

738 **Table 1: Overview of samples.**

739 Specimen numbers and species identities are given as in the 16S phylogeny (Figure 2).  
740 “Genbank” indicates to the Genbank submission numbers of each specimen and marker (16S  
741 or 28S). Locality codes and names refer to sample localities during the four expeditions on  
742 Lake Baikal, except for the locality in the UK. S=south. Lat= latitude, Long=longitude.

743

744 **Table 2: Results of species delimitations with the PTP and the 4  $\theta$  method from the**  
745 **congruent molecular dataset.**

746 Results of the PTP algorithm are given in italics for each phylogenetic clade in the diagonal  
747 line, the results of the 4  $\theta$  rule for comparisons between phylogenetic clades are shown below  
748 the diagonal. Phylogenetic clades printed in bold fulfil the criteria of both methods. *t=*  
749 *tuberculata*.

750 For the PTP method, the statistical support for each particular phylogenetic group of  
751 individuals is provided. For the 4  $\theta$  rule, only the ratio for the genetic distance between  
752 phylogenetic clades is shown here; other calculations on which these ratios are based, are  
753 detailed in Table S1 (supplementary material).

754 \* = Singletons, to which the 4  $\theta$  method cannot be applied. \$ = Additional evolutionary  
755 genetic species detected with the PTP method. £ = Additional evolutionary genetic species  
756 detected with the 4  $\theta$  rule.

757

758 **Table 3: Ecological and geographical distribution data of genetic species from the**  
759 **congruent dataset.**

760 Genetic species and their pairs were defined from well-supported clades in the combined  
761 phylogenetic trees (using congruent sequence data from both 16S and 28S; Figure 2).  
762 Distribution data differentiating sister pairs are printed in bold. na= no data available;  
763 N=North, E=Eastern, C= Central, S=Southern, W= Western. Depth is water depth in meters.  
764

## 765 **Figures**

### 766 **Figure 1: Approximate position of sampling stations in Lake Baikal.**

767 Labels refer to the sampling stations in Table 1.

768

### 769 **Figure 2: Congruent phylogeny based on 16S and 28S.**

770 This phylogeny has been constructed with Maximum Likelihood and Bayesian methods on  
771 DNA sequences of 922 nucleotides each. Statistical support is shown above (PHYML,  
772 bootstrap values) and below (MrBayes, posterior probabilities) branches, respectively. *C. sp.*  
773 1 to *C. sp.* 4 are new species still awaiting formal description. Roman numbers refer to  
774 genetic species according to Table 2. Species printed in bold also show morphological  
775 differences. The coloured columns next to the tree show the type of sediment (A), depth (B),  
776 basin (C) and shore (D). Missing data are indicated in black.

777

### 778 **Figure 3: Results of the Principal Coordinate Analysis of genetic species defined by the** 779 **congruent molecular dataset and their ecological and geographic distributions.**

780 Identities of genetic species are similar to Figure 2. Species pairs from Table 3 are indicated  
781 by similar colours. Unpaired species are shown in black. If dots are labelled with several  
782 species names, these species share the same space in the coordination system. For all species  
783 shown, ecological data on sediment type and water depth were taken from Mazepova (1998;

784 see Table S2 for details), and geographical distribution data according to Table 3. Note that  
785 the new species *C. sp. 4* from Table 3 was not included in the analyses because no data on  
786 sediment type and water depth were available for this species from Mazepova (1998). *t* =  
787 *tuberculata*.

788

789 **Supplementary material:**

790 **Table S1: Results of evolutionary species delimitations with the 4  $\theta$  rule.**

791 Specimen numbers and species names refer to the 16S phylogeny (Figure 2).  $n^1$  = number of  
792 specimens for sister clade 1,  $n^2$  number of specimens for sister clade 2.  $\theta$  corresponds to the  
793 population mutation rate, having been corrected for the number of specimens.  $D$ = genetic  
794 distance.  $na$ = not applicable - calculations could not be conducted because only one sequence  
795 (singleton) was present per phylogenetic sister clade. Ratios of  $D/\theta$  larger than 4 fulfil the  
796 criteria of the 4 theta rule and are indicated in bold.

797

798 **Table S2: Comparison of ecological distribution data between our dataset and the data**  
799 **from Mazepova (1998).**

800 For the newly described species *C. sp. 1* to *C. sp. 4*, no data were available from Mazepova  
801 (1998).

802

803 **Table S3: Results of species delimitations with the PTP and the 4  $\theta$  method from 16S.**

804 Results of the PTP algorithm are given in italics for each phylogenetic clade in the diagonal  
805 line, the results of the 4  $\theta$  rule for comparisons between phylogenetic clades are shown below  
806 the diagonal. Phylogenetic clades printed in bold fulfil the criteria of both methods.  $t$ =  
807 *tuberculata*. For the PTP method, the statistical support for each particular phylogenetic  
808 group of individuals is provided. For the 4  $\theta$  rule, only the ratio for the genetic distance

809 between phylogenetic clades is shown here; other calculations on which these ratios are  
810 based, are available from IS on request. \* = Singletons, to which the 4  $\theta$  method cannot be  
811 applied. \$ = Additional evolutionary genetic species detected with the PTP method. £ =  
812 Additional evolutionary genetic species detected with the 4  $\theta$  rule.

813

814 **Figure S1: 16S phylogeny.**

815 Statistical support is shown above (PHYML, bootstrap values) and below (MrBayes,  
816 posterior probabilities) branches, respectively. *C. sp. 1* to *C. sp. 4* are new species still  
817 awaiting formal description. Roman numbers refer to genetic species according to Table 2.  
818 Species printed in bold also show morphological differences. Specimens being also present in  
819 the combined 16S/28S phylogeny are indicated with a plus. The coloured columns next to the  
820 tree show the type of sediment (A), depth (B), basin (C) and shore (D). Missing data are  
821 indicated in black.

822

823 **Figure S2: 28S phylogeny.**

824 Statistical support is shown above branches as bootstrap values of 100%. Bayesian methods  
825 did not support the phylogeny whatsoever. Species names and Roman numbers refer to the  
826 species as delimited by 16S (Figure S2). For underlined specimens, no 16S data are  
827 available.

828

829 **Figure S3: Illustrations of valve morphology of the five genetic species within the classic**  
830 **morpho-species *Cytherissa tuberculata tuberculata*.**

831 A,B : *C. tuberculata tuberculata* I (loc. BK15). C,D : *C. tuberculata tuberculata* II (loc.  
832 BK8). E,F: *C. tuberculata tuberculata* III (loc. BT11). G,H : *C. tuberculata tuberculata* IV  
833 (loc. BK21). I,J : *C. tuberculata tuberculata* V (loc. BT18).

834 A, C, E, G, I: Right Valves, external views. B, D, F, H, J: Right Valves, internal views.  
835 Scales: 1 mm for E, G, I. 500  $\mu$ m for A, B, C, D, F, H, J. Please note that genetic species *C.*  
836 *tuberculata tuberculata* I can only be recognized by 16S (Table 1 & S3; Figure S1 & S4).

837

838 **Figure S4: 16S minimum spanning network.**

839 For each haplotype, the size of the circle is proportional to its frequency. Colours indicate the  
840 geographic origin of haplotypes from the three Baikalian basins and the UK, respectively.  
841 Species identities of haplotypes are indicated by coloured names and circles in the same  
842 colours around haplotypes. Species names refer to Figure S1.

843

844

845 **Figure S5: 28S minimum spanning network.**

846 For each haplotype, the size of the circle is proportional to its frequency. Colours indicate the  
847 geographic origin of haplotypes from the three Baikalian basins and the UK, respectively.  
848 Species identities of haplotypes are indicated by coloured names and circles in the same  
849 colours around haplotypes. Species names refer to Figure S2 or, if underlined, to the  
850 identification *sensu* Mazepova (1990) because no 16S data are available for these specimens.  
851 If several species names are shown next to a haplotype, then the latter is shared between these  
852 species. The most common haplotype is found in 20 different species.

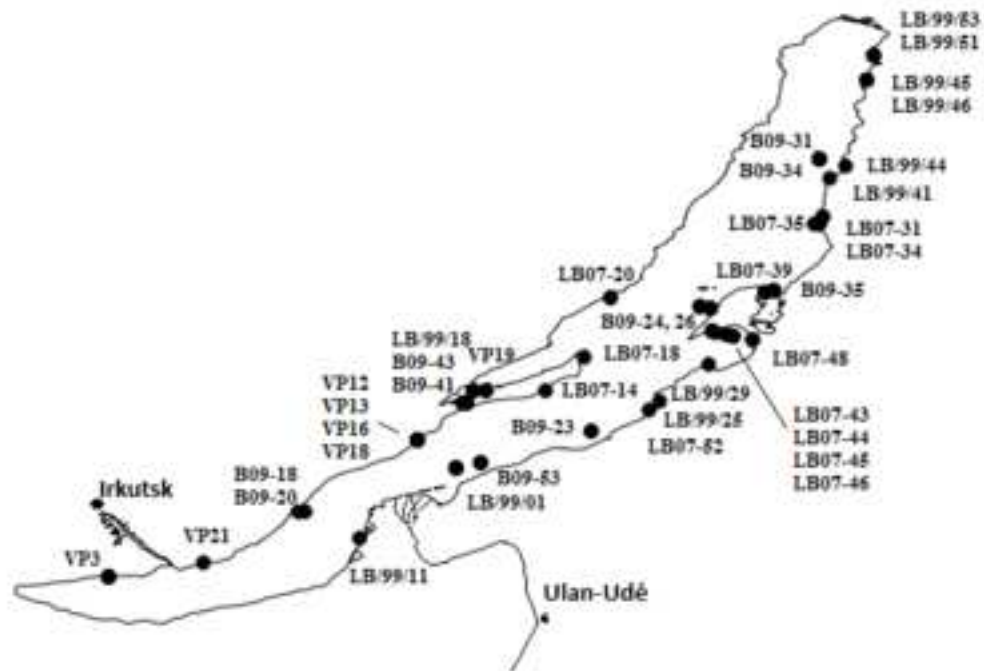




Figure2

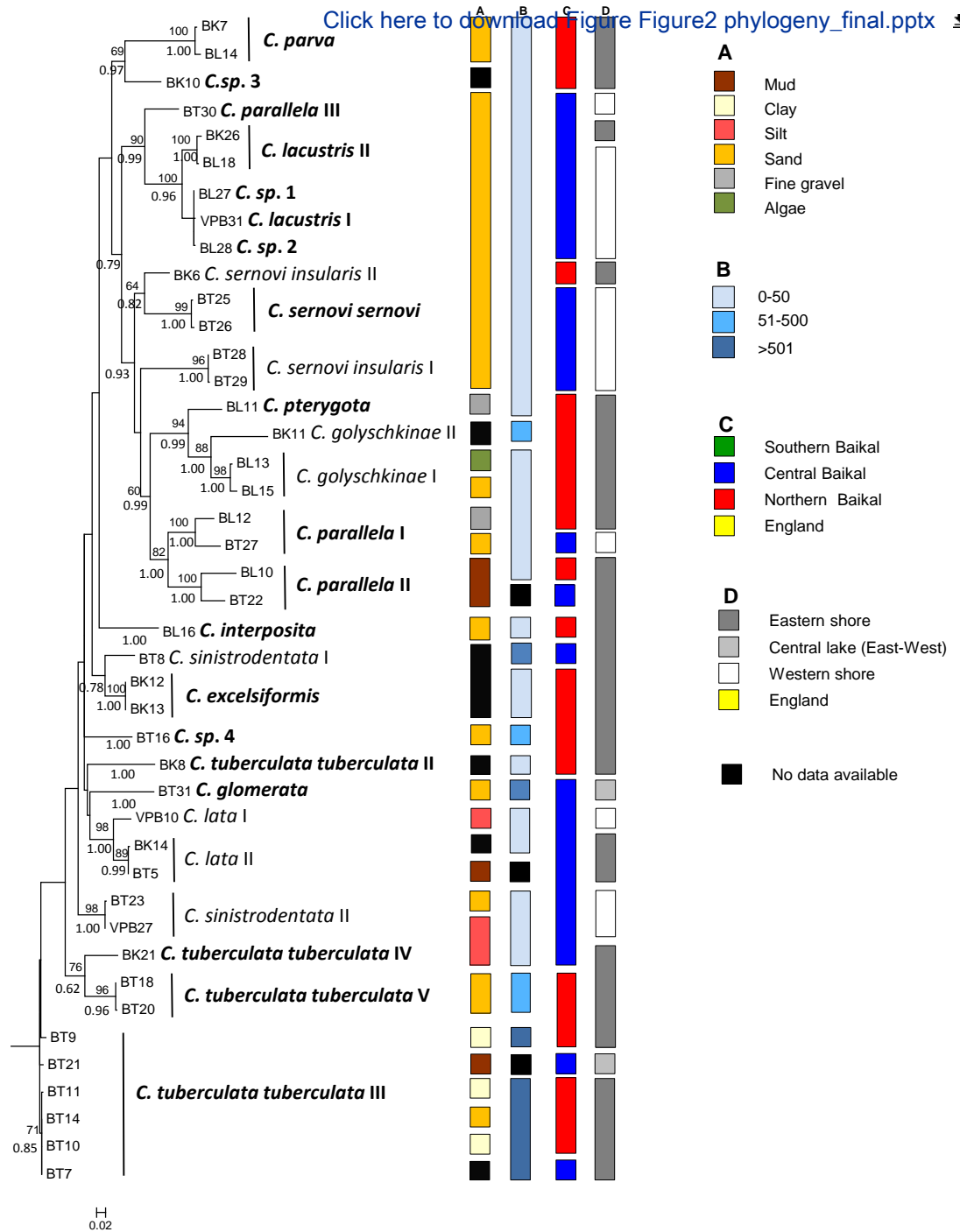
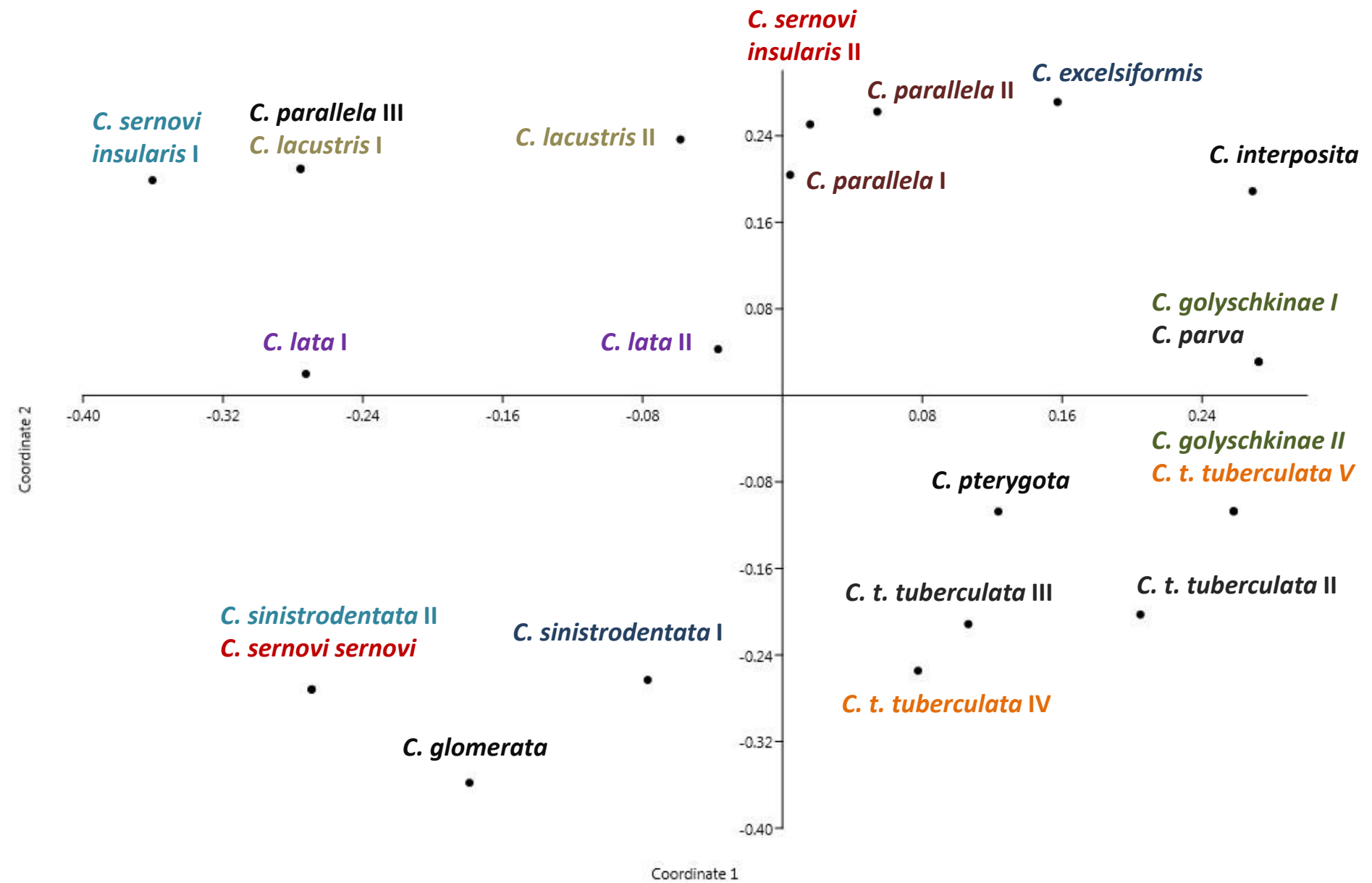


Figure 3



**Table 1: Overview of samples.**

Specimen numbers and species identities are given as in the 16S phylogeny (Figure 2). Genbank refers to the genbank numbers of each specimen and marker (16S or 28S). Locality codes and names refer to sample localities during the four expeditions on Lake Baikal, except for the locality in the UK. S=south. Lat= latitude, Long=longitude.

Specimen	Genbank 16S	Genbank 28S	Species	Locality code	Locality name	Lat	Long
BK1		X	<i>C. parva</i>	LB07-14	Olkhon island	53.1269	107.4868
BK3		X	<i>C. parallela</i>	LB07-18	Uzury	53.3592	107.7698
BK4		X	<i>C. parallela</i>	LB07-20	Kocherikovskiy cape	53.7795	107.9465
BK5		X	<i>C. parallela</i>	LB07-20	Kocherikovskiy cape	53.7795	107.9465
BK6	X	X	<i>C. sernovi insularis</i> II	LB07-31	Davcha Bay	54.3611	109.4709
BK7	X	X	<i>C. parva</i>	LB07-31	Davcha Bay	54.3611	109.4709
BK8	X	X	<i>C. tuberculata tuberculata</i> II	LB07-34	Davcha Bay	54.3101	109.4546
BK10	X	X	<i>C. sp.3</i>	LB07-34	Davcha Bay	54.3101	109.4546
BK11	X	X	<i>C. golyschkiniae</i> II	LB07-35	Davcha Bay	54.3183	109.4105
BK12	X	X	<i>C. excelsiformis</i>	LB07-39	Chivirkuy Bay	53.8255	109.0546
BK13	X	X	<i>C. excelsiformis</i>	LB07-39	Chivirkuy Bay	53.8255	109.0546
BK14	X	X	<i>C. lata</i> II	LB07-43	Barguzin Bay	53.5547	108.6814
BK15	X		<i>C. tuberculata tuberculata</i> I	LB07-43	Barguzin Bay	53.5547	108.6814

BK16		X	<i>C. tuberculata tuberculata</i>	LB07-43	Barguzin Bay	53.5547	108.6814
BK19	X		<i>C. tuberculata tuberculata</i> III	LB07-44	Barguzin Bay	53.5419	108.6987
BK20	X		<i>C. sp.4</i>	LB07-45	Barguzin Bay	53.5259	108.7770
BK21	X	X	<i>C. tuberculata tuberculata</i> IV	LB07-45	Barguzin Bay	53.5259	108.7770
BK22	X		<i>C. sinistrodentata</i> I	LB07-46	Barguzin Bay	53.5173	108.8256
BK23	X		<i>C. lata</i> II	LB07-46	Barguzin Bay	53.5173	108.8256
BK25	X		<i>C. lacustris</i> II	LB07-48	Barguzin Bay	53.4803	108.9635
BK26	X	X	<i>C. lacustris</i> II	LB07-48	Barguzin Bay	53.4803	108.9635
BK28	X		<i>C. pterygota</i>	LB07-52	Goryachinsk	52.9839	108.2299
BL3	X		<i>C. verrucosa</i>	LB/99/51	Frolika Bay. S corner	55.5217	109.8453
BL10	X	X	<i>C. cf. parallela</i> II	LB/99/51	Frolika Bay. S corner	55.5217	109.8453
BL11	X	X	<i>C. pterygota</i>	LB/99/45	S part of Khakusy Bay	55.3497	109.7861
BL12	X	X	<i>C. parallela</i> I	LB/99/45	S part of Khakusy Bay	55.3497	109.7861
BL13	X	X	<i>C. golyschkiniae</i> I	LB/99/44	Severny Birakan	54.7394	109.6394
BL14	X	X	<i>C. parva</i>	LB/99/53	Frolika Bay. S corner	55.5217	109.8453
BL15	X	X	<i>C. golyschkiniae</i> I	LB/99/41	3 km N of Kabanij Cape	54.6500	109.5278
BL16	X	X	<i>C. interposta</i>	LB/99/46	S part of Khakusy Bay	55.3497	109.7861
BL17		X	<i>C. lacustris</i>	LB/99/01	Selenga delta. S of	52.5722	106.8500

					middle arm		
BL18	X	X	<i>C. lacustris</i> II	LB/99/01	Selenga delta. S of middle arm	52.5722	106.8500
BL19		X	<i>C. lacustris</i>	LB/99/01	Selenga delta. S of middle arm	52.5722	106.8500
BL20		X	<i>C. parva</i>	LB/99/53	Frolika Bay. S corner	55.5217	109.8453
BL21		X	<i>C. lacustris</i>	LB/99/01	Selenga delta. S of middle arm	52.5722	106.8500
BL22		X	<i>C. lacustris</i>	LB/99/01	Selenga delta. S of middle arm	52.5722	106.8500
BL23		X	<i>C. tuberculata tuberculata</i>	LB/99/25	Bezemyannaya Bay	53.0528	108.3133
BL24		X	<i>C. parva</i>	LB/99/53	Frolika Bay. S corner	55.5217	109.8453
BL26		X	<i>C. verrucosa</i>	LB/99/29	Barguzin Bay. opposite Cape Kholodyanka	53.3142	108.6564
BL27	X	X	<i>C. sp.1</i>	LB/99/18	Ogoi Island. Male Morje	53.1316	106.9790
BL28	X	X	<i>C. sp.1</i>	LB/99/18	Ogoi Island. Male Morje	53.1316	106.9790
BL32		X	<i>C. tuberculata tuberculata</i>	LB/99/11	Selenga delta. opposite Posol'ski village	52.0672	106.1656
BT1		X	<i>C. sp.6</i>	B09-18	Peschanaya Bay	52.2617	105.7189

BT2		X	<i>C. tuberculata tuberculata</i>	B09-20	in front of Peschanaya Bay	52.2670	105.7736
BT3		X	<i>C. tuberculata tuberculata</i>	B09-23	Near Gremyachinsk town	52.8329	107.8130
BT4		X	<i>C. tuberculata tuberculata</i>	B09-23	Near Gremyachinsk town	52.8329	107.8130
BT5	X	X	<i>C. lata</i> II	B09-24	Near Svyatoy Nos (The Holy Nose) peninsula	53.7220	108.6010
BT6		X	<i>C. tuberculata tuberculata</i>	B09-26	Near Svyatoy Nos (The Holy Nose) peninsula	53.7080	108.6660
BT7	X	X	<i>C. tuberculata tuberculata</i> III	B09-26	Near Svyatoy Nos (The Holy Nose) peninsula	53.7080	108.6660
BT8	X	X	<i>C. sinistrodentata</i> I	B09-26	Near Svyatoy Nos (The Holy Nose) peninsula	53.7080	108.6660
BT9	X	X	<i>C. tuberculata tuberculata</i> III	B09-31	Near Urbikan cape	54.7875	109.4422
BT10	X	X	<i>C. tuberculata tuberculata</i> III	B09-31	Near Urbikan cape	54.7875	109.4422
BT11	X	X	<i>C. tuberculata tuberculata</i> III	B09-31	Near Urbikan cape	54.7875	109.4422
BT12		X	<i>C. tuberculata tuberculata</i>	B09-31	Near Urbikan cape	54.7875	109.4422
BT13		X	<i>C. tuberculata tuberculata</i>	B09-34	Near Kabaniy cape	54.6646	109.3927
BT14	X	X	<i>C. tuberculata tuberculata</i> III	B09-34	Near Kabaniy cape	54.6646	109.3927
BT15		X	<i>C. sp.4</i>	B09-35	Near Chivyrkuy bay	53.8363	109.1185

BT16	X	X	<i>C. sp.4</i>	B09-35	Near Chivyrkuy bay	53.8363	109.1185
BT17		X	<i>C. sp.4</i>	B09-35	Near Chivyrkuy bay	53.8363	109.1185
BT18	X	X	<i>C. tuberculata tuberculata</i> V	B09-35	Near Chivyrkuy bay	53.8363	109.1185
BT19		X	<i>C. tuberculata tuberculata</i>	B09-35	Near Chivyrkuy bay	53.8363	109.1185
BT20	X	X	<i>C. tuberculata tuberculata</i> V	B09-35	Near Chivyrkuy bay	53.8363	109.1185
BT21	X	X	<i>C. tuberculata tuberculata</i> III	B09-24	Near Svyatoy Nos (The Holy Nose) peninsula	53.7220	108.6010
BT22	X	X	<i>C. cf. parallela</i> II	B09-24	Near Svyatoy Nos (The Holy Nose) peninsula	53.7220	108.6010
BT23	X	X	<i>C. sinistrodentata</i> II	B09-41	Olkhon gate strait	53.0308	106.9226
BT24		X	<i>C. sernovi sernovi</i>	B09-41	Olkhon gate strait	53.0308	106.9226
BT25	X	X	<i>C. sernovi sernovi</i>	B09-41	Olkhon gate strait	53.0308	106.9226
BT26	X	X	<i>C. sernovi sernovi</i>	B09-41	Olkhon gate strait	53.0308	106.9226
BT27	X	X	<i>C. parallela</i> I	B09-43	Olkhon gate strait	53.0384	106.9024
BT28	X	X	<i>C. sernovi insularis</i> I	B09-43	Olkhon gate strait	53.0384	106.9024
BT29	X	X	<i>C. sernovi insularis</i> I	B09-43	Olkhon gate strait	53.0384	106.9024
BT30	X	X	<i>C. parallela</i> III	B09-43	Olkhon gate strait	53.0384	106.9024
BT31	X	X	<i>C. glomerata</i>	B09-53	Near Suchaya village	52.5996	107.0218
BT32	X		<i>C. tuberculata tuberculata</i> III	B09-53	Near Suchaya village	52.5996	107.0218

BT33	X	X	<i>C. tuberculata tuberculata</i> III	B09-53	Near Suchaya village	52.5996	107.0218
VPB3		X	<i>C. sernovi insularis</i>	VP3	Polovinnaya	51.7956	104.3665
VPB4		X	<i>C. tuberculata tuberculata</i>	VP12	Bay Anga	52.7780	106.5834
VPB5		X	<i>C. sp.6</i>	VP13	Bay Anga	52.7780	106.5834
VPB8		X	<i>C. tuberculata tuberculata</i>	VP19	Semesosennaya	53.1246	107.0622
VPB9	X		<i>C. parallela</i> III	VP19	Semesosennaya	53.1246	107.0622
VPB10	X	X	<i>C. lata</i> I	VP16	Bay Anga	52.7780	106.5834
VPB11		X	<i>C. sinistrodentata</i>	VP16	Bay Anga	52.7780	106.5834
VPB14	X		<i>C. cf. golyschkiniae</i> IV	VP3	Polovinnaya	51.7956	104.3665
VPB15	X		<i>C. cf. golyschkiniae</i> IV	VP3	Polovinnaya	51.7956	104.3665
VPB16	X		<i>C. cf. golyschkiniae</i> IV	VP3	Polovinnaya	51.7956	104.3665
VPB17	X		<i>C. golyschkiniae</i> III	VP3	Polovinnaya	51.7956	104.3665
VPB18	X		<i>C. golyschkiniae</i> III	VP3	Polovinnaya	51.7956	104.3665
VPB20	X		<i>C. sp.2</i>	VP21	Bay Bolshiye Katy	51.9005	105.0766
VPB22	X		<i>C. sinistrodentata</i> II	VP18	Bay Anga	52.7780	106.5834
VPB23	X		<i>C. sinistrodentata</i> II	VP18	Bay Anga	52.7780	106.5834
VPB24	X		<i>C. sernovi sernovi</i>	VP18	Bay Anga	52.7780	106.5834
VPB25	X		<i>C. sernovi sernovi</i>	VP18	Bay Anga	52.7780	106.5834



VPB26		X	<i>C. sernovi sernovi</i>	VP18	Bay Anga	52.7780	106.5834
VPB27	X	X	<i>C. sinistrodentata</i> II	VP18	Bay Anga	52.7780	106.5834
VPB28		X	<i>C. sernovi sernovi</i>	VP18	Bay Anga	52.7780	106.5834
VPB29	X		<i>C. lata</i> I	VP18	Bay Anga	52.7780	106.5834
VPB30		X	<i>C. lacustris</i>	VP13	Bay Anga	52.7780	106.5834
VPB31	X	X	<i>C. lacustris</i> I	VP13	Bay Anga	52.7780	106.5834
CL1	X		<i>C. lacustris</i> II		Semerwater, UK	54.2806	-002.1250
CL14	X		<i>C. lacustris</i> II		Semerwater, UK	54.2806	-002.1250
CL15		X	<i>C. lacustris</i>		Semerwater, UK	54.2806	-002.1250
CL16		X	<i>C. lacustris</i>		Semerwater, UK	54.2806	-002.1250
CL17		X	<i>C. lacustris</i>		Semerwater, UK	54.2806	-002.1250
CL18		X	<i>C. lacustris</i>		Semerwater, UK	54.2806	-002.1250
CL19		X	<i>C. lacustris</i>		Semerwater, UK	54.2806	-002.1250
Outgroup	KF061156	KF061166	<i>Romecytheridea ampla</i>		Lake Tangayika, Tanzania		

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**Table 2: Results of species delimitations with the PTP and the 4 theta method combining 16S and 28S data.**

We show here only details of the species delimitations within morphospecies that could potentially be cryptic. Morphological (sub)species *sensu* Mazepova (1990) and the four newly discovered morphospecies (*C. sp.* 1 to *C. sp.* 4) all have clear morphological differences, and species boundaries are supported by the three methods delimitating genetic species. Detailed results are therefore not shown for these morphospecies but are available on request. Results of the PTP algorithm are given in italics for each phylogenetic clade in the diagonal line, the results of the 4  $\theta$  rule for comparisons between phylogenetic clades are shown below the diagonal. Phylogenetic clades printed in bold fulfil the criteria of both methods. For the PTP method, the statistical support for each particular phylogenetic group of individuals is provided (maximal value is 1.0). For the 4  $\theta$  rule, values shown are the ratios of genetic distances between phylogenetic clades that are needed to be larger than 4 to delimitate genetic species. The values from which these ratios have been calculated are detailed in Table S1 (supplementary material). . t= *tuberculata*. \* Singleton, to which the 4  $\theta$  method cannot be applied. \$ additional evolutionary genetic species detected with the PTP method.

	<i>C. t. tuberculata</i> II	<i>C. t. tuberculata</i> III	<i>C. t. tuberculata</i> IV	<i>C. t. tuberculata</i> V
<b><i>C. tuberculata tuberculata</i> II</b>	<i>1.00</i>			
<b><i>C. tuberculata tuberculata</i> III</b>	39.11	<i>0.91</i>		
<b><i>C. tuberculata tuberculata</i> IV</b>	*	35.20	<i>1.00</i>	
<b><i>C. tuberculata tuberculata</i> V</b>	127.92	35.75	72.90	<i>1.00</i>
	<i>C. parallela</i> I	<i>C. parallela</i> II	<i>C. parallela</i> III	
<b><i>C. parallela</i> I</b>	<i>0.99 (IA<sup>\$</sup>) 0.99 (IB<sup>\$</sup>)</i>			
<b><i>C. parallela</i> II</b>	4.16	<i>1.00 (IIA<sup>\$</sup>) 1.00 (IIB<sup>\$</sup>)</i>		
<b><i>C. parallela</i> III</b>	5.59	4.91	<i>1.00</i>	
	<i>C. golyschkiniae</i> I	<i>C. golyschkiniae</i> II		
<b><i>C. golyschkiniae</i> I</b>	<i>0.84</i>			
<b><i>C. golyschkiniae</i> II</b>	13.21	<i>1.00</i>		
	<i>C. sernovi insularis</i> I	<i>C. sernovi insularis</i> II		
<b><i>C. sernovi insularis</i> I</b>	<i>1.00</i>			
<b><i>C. sernovi insularis</i> II</b>	98.63	<i>0.86</i>		
	<i>C. lacustris</i> I	<i>C. lacustris</i> II		
<b><i>C. lacustris</i> I</b>	<i>0.83</i>			
<b><i>C. lacustris</i> II</b>	9.43	<i>0.90</i>		
	<i>C. sinistrodentata</i> I	<i>C. sinistrodentata</i> II		
<b><i>C. sinistrodentata</i> I</b>	<i>1.00</i>			
<b><i>C. sinistrodentata</i> II</b>	57.69	<i>0.86</i>		

	<i>C. lata</i> I	<i>C. lata</i> II
<i>C. lata</i> I	0.98	
<i>C. lata</i> II	38.46	0.98

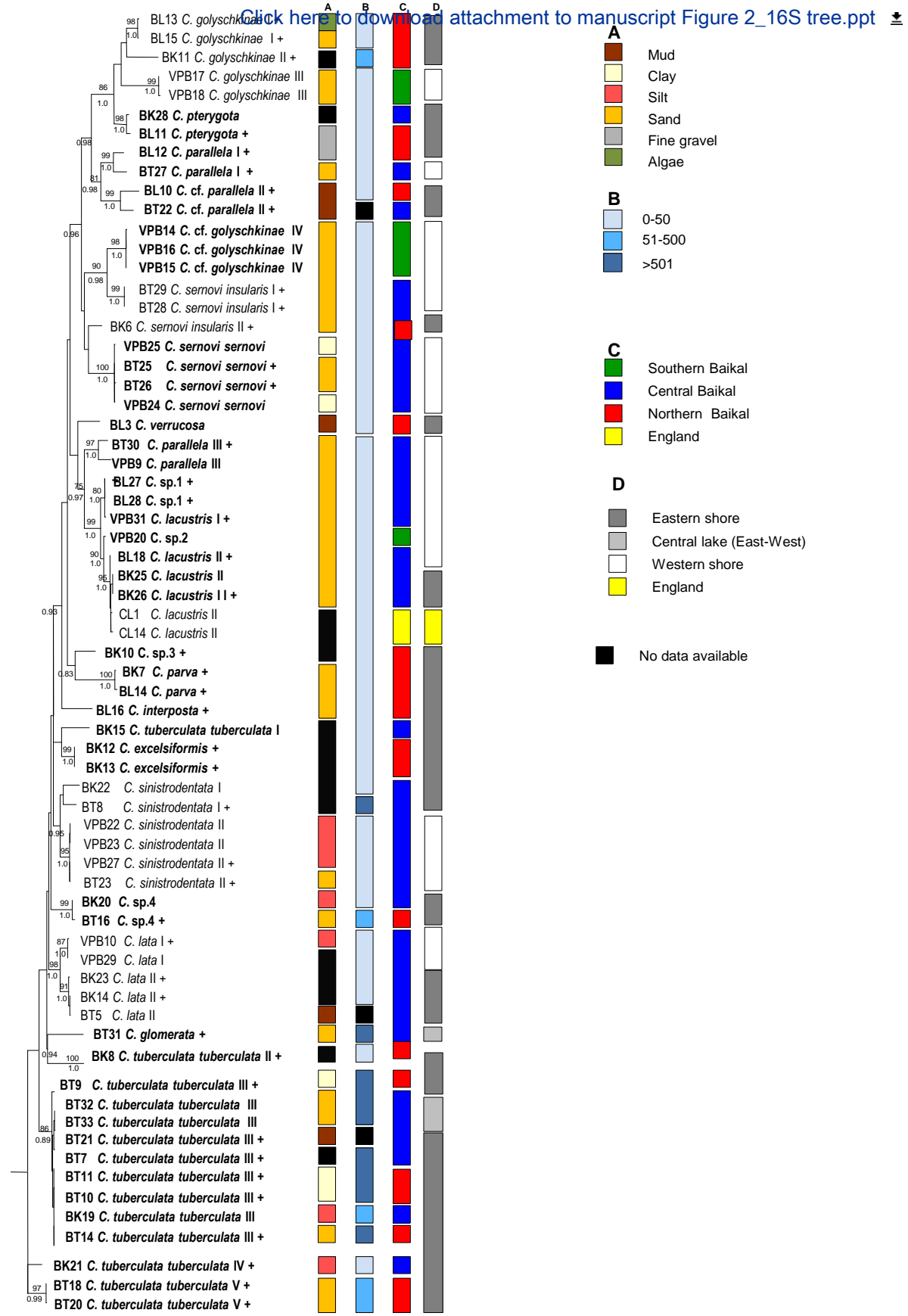
**Table 3: Current ecological and geographic distribution of genetic *Cytherissa* species.**

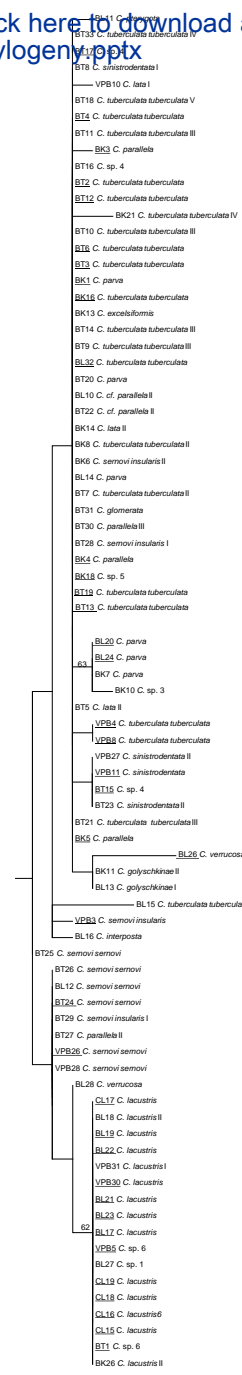
Genetic species and their pairs were defined from well-supported clades in the combined phylogenetic trees (using congruent sequence data from both 16S and 28S; Figure 2). Distribution data differentiating sister pairs are printed in bold. na= no data available; N=North, E=Eastern, C= Central, S=Southern, W= Western. Depth is water depth in meters.

Genetic species	Sediment	Depth	Basin	Shore
<i>C. golyschkiniae</i> I	Algae/Sand	0-50	N	E
<i>C. golyschkiniae</i> II	na	<b>51-500</b>	N	E
<i>C. parallela</i> I	Fine gravel/sand	0-50	C, N	E, W
<i>C. cf. parallela</i> II	<b>Mud</b>	0-50/na	C, N	E
<i>C. sernovis insularis</i> II	Sand	0-50	C	E
<i>C. sernovi sernovi</i>	Clay/Sand	0-50	C	<b>W</b>
<i>C. lacustris</i> I	Mud	0-50	C	<b>W</b>
<i>C. lacustris</i> II	Mud/na	0-50	C	E
<i>C. parva</i>	Mud	0-50	N	E
<i>C. sp. 3</i>	na	0-50	N	E
<i>C. sinistrodentata</i> I	na	0-50, > <b>501</b>	C	E
<i>C. excelsiformis</i>	na	0-50	N	E
<i>C. lata</i> I	Silt/na	0-50	C	<b>W</b>
<i>C. lata</i> II	<b>Mud</b> /na	0-50, na	C	E
<i>C. tuberculata tuberculata</i> IV	Silt	0-50	C	E
<i>C. tuberculata tuberculata</i> V	<b>Sand</b>	<b>51-500</b>	N	E
<i>C. sernovis insularis</i> I	Sand	0-50	C	W
<i>C. sinistrodentata</i> II	Silt/Mud	0-50	C	<b>W</b>
<i>C. glomerata</i>	Sand	> 501	C	C
<i>C. interposita</i>	Sand	0-50	N	E
<i>C. parallela</i> III	Mud	0-50	C	W
<i>C. pterygota</i>	Fine gravel/na	0-50	C, N	E
<i>C. sp. 4</i>	Sand/Mud	0-50, 51-500	C, N	C, E
<i>C. tuberculata tuberculata</i> II	na	0-50	N	E
<i>C. tuberculata tuberculata</i> III	Sand/Clay/Silt/Mud/na	51-500, >501, na	C, N	E



Figure S1





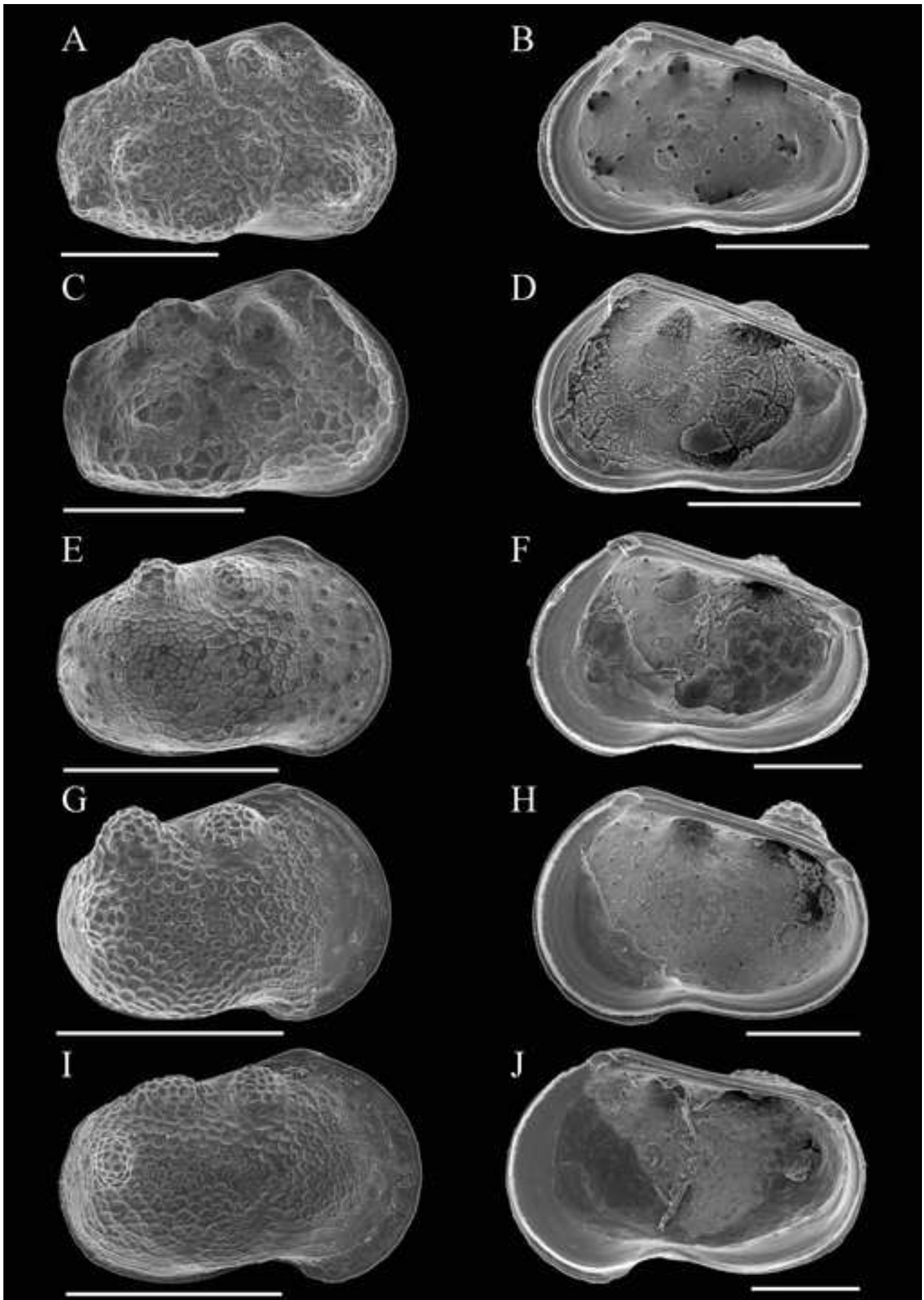




Figure S4

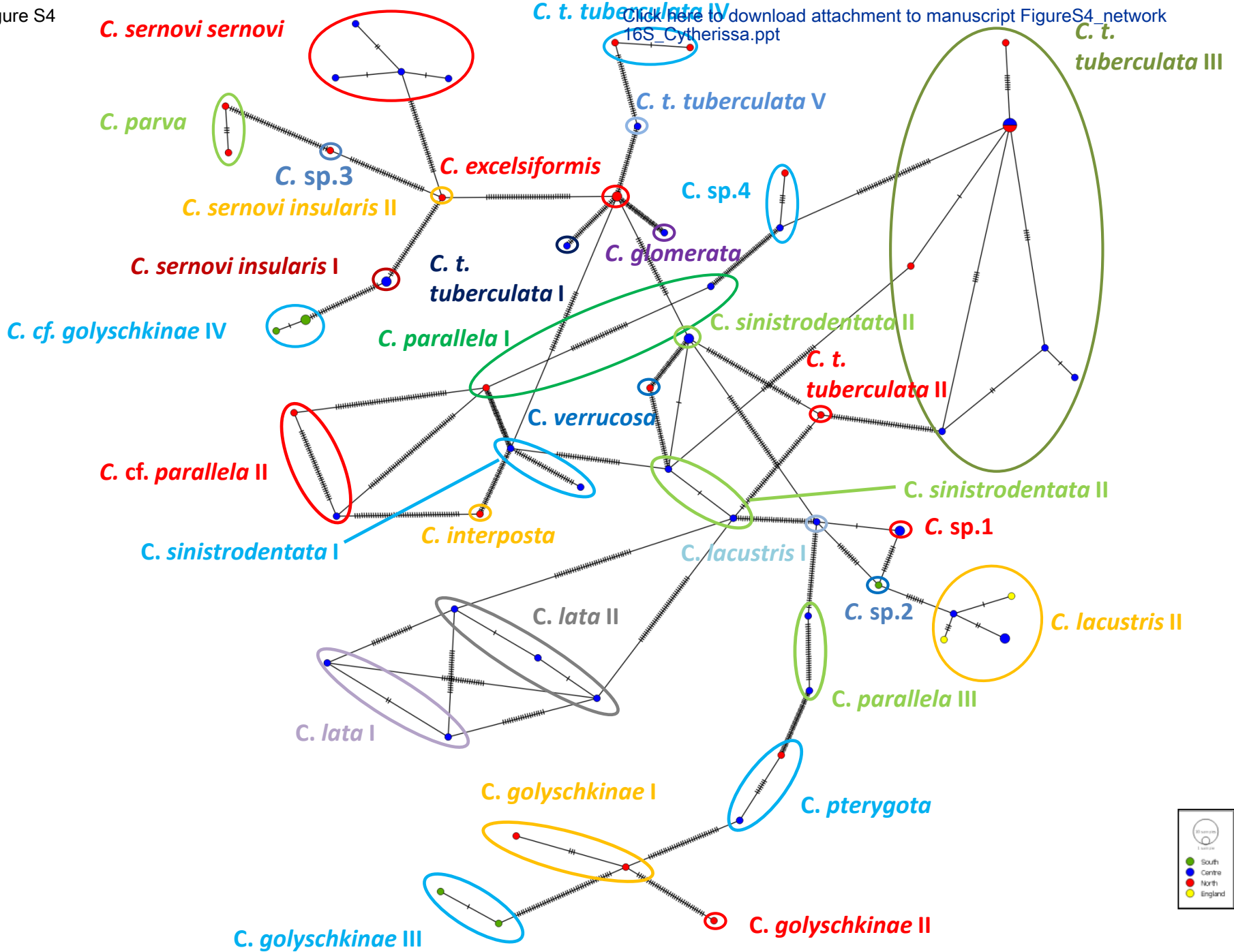
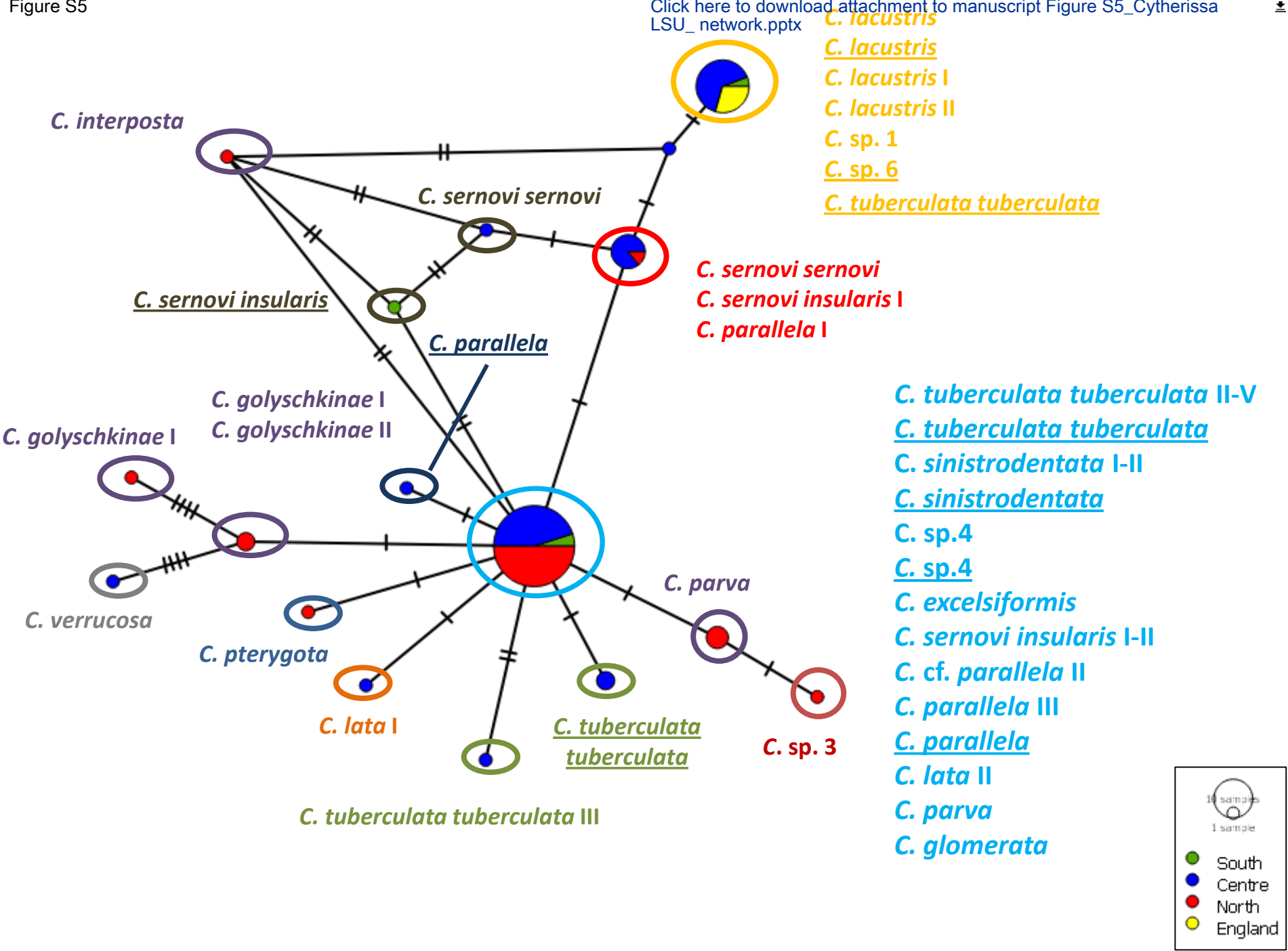


Figure S5

Click here to download attachment to manuscript Figure S5\_Cytherissa  
 LSU\_network.pptx



**Table S1: Results of evolutionary species delimitations with the 4 theta rule (Birky et al. 2010).**

Specimen numbers and species names refer to the phylogeny from combined molecular data (Figure 2).  $n^1$  = number of specimens for sister clade 1,  $n^2$  number of specimens for sister clade 2.  $\theta$  corresponds to the Watterson estimate of population genetic variability. D= genetic distance. na= non applicable - calculations could not be conducted because only one sequence (singleton) was present per phylogenetic sister clade. Ratios of D/ $\theta$  larger than 4 fulfil the criteria of the 4 theta rule and are indicated in bold.

Specimens of sister clades	Species	$n^1$ $n^2$	$\theta$ (within clades)	D (between clades)	Ratio D/ $\theta$
BL13, BL15; BK11	<i>C. golyschkiniae I</i> <i>C. golyschkiniae II</i>	2 1	0.0049 na	0.065	<b>13.21</b>
BL12, BT27; BL10, BT22	<i>C. parallela I</i> <i>C. parallela II</i>	2 2	0.0177 0.0200	0.083	4.16
BL12, BT27; BT30, VPB9	<i>C. parallela I</i> <i>C. parallela III</i>	2 1	0.0177 na	0.099	<b>5.59</b>
BL10, BT22; BT30, VPB9	<i>C. parallela II</i> <i>C. parallela III</i>	2 2	0.0200 na	0.098	4.91
BT29, BT28 ; BK6	<i>C. sernovi insularis I</i> <i>C. sernovi insularis II</i>	2 1	0.0007 na	0.072	<b>98.63</b>
BL27, BL28, VB31; VBP20	<i>C. lacustris I</i> <i>C. lacustris II</i>	3 2	0.0007 0.0029	0.027	<b>9.43</b>
BK8; BT9; BT21, BT11, BT14, BT10, BT7	<i>C. tuberculata II</i> <i>C. tuberculata III</i>	1 6	na 0.0018	0.070	<b>39.11</b>
BK8; BK21	<i>C. tuberculata II</i> <i>C. tuberculata IV</i>	1 1	na na	0.096	<b>na</b>
BK8;	<i>C. tuberculata II</i>	1	na	0.093	<b>127.92</b>

BT18, BT20	<i>C. tuberculata V</i>	2	0.0007		
BT9; BT21, BT11, BT14, BT10, BT7	<i>C. tuberculata III</i>	6	0.0018	0.063	<b>35.20</b>
BK21	<i>C. tuberculata IV</i>	1	na		
BT9; BT21, BT11, BT14, BT10, BT7	<i>C. tuberculata III</i>	6	0.0018	0.064	<b>35.75</b>
BT18, BT20	<i>C. tuberculata V</i>	2	0.0007		
BK21;	<i>C. tuberculata IV</i>	1	na	0.053	<b>72.90</b>
BT18, BT20	<i>C. tuberculata V</i>	2	0.0007		
BT8;	<i>C. sinistrodentata I</i>	1	na	0.042	<b>57.69</b>
BT23, VPB27	<i>C. sinistrodentata II</i>	2	0.0007		
VPB10;	<i>C. lata I</i>	1	na	0.028	<b>38.46</b>
BK14, BT5	<i>C. lata II</i>	2	0.0007		

**Table S2: Comparison between our ecological data and the data of Mazepova (1998).**

This table shows data from the genetic species that were defined from the congruent molecular data set (Table 4) and the morphospecies *sensu* Mazepova (1990) and their distributions (Mazepova, 1998). na= no data available; differences are printed in bold. Depth represents water depth in meters. Please note that no ecological distribution data are available from Mazepova (1998) for the new species *C. sp. 1* to *C. sp. 4*.

Genetic species	<b>Sediment</b> - our data Mazepova (1998)	<b>Depth</b> - our data Mazepova (1998)
<i>C. golyschkiniae</i> I	Algae/Sand <b>All types</b>	0-50 1-50
<i>C. golyschkiniae</i> II	na All types	<b>51-500</b> <b>1-50</b>
<i>C. parallela</i> I	Fine gravel/sand Sand	0-50 1-70
<i>C. cf. parallela</i> II	Mud <b>Sand</b>	0-50/na 1-70
<i>C. parallela</i> III	Mud <b>Sand</b>	0-50 1-70
<i>C. sernovis insularis</i> I	Sand Sand	0-50 0-20
<i>C. sernovis insularis</i> II	Sand Sand	0-50 0-20
<i>C. sernovi sernovi</i>	Clay/Sand Sand/Silts	0-50 <b>100-1000</b>
<i>C. lacustris</i> I	Mud <b>Sand</b>	0-50 0-50
<i>C. lacustris</i> II	Mud/na <b>Sand</b>	0-50 0-50
<i>C. lata</i> I	Silt/na <b>Soft types</b>	0-50 0-100
<i>C. lata</i> II	Mud/na <b>Soft types</b>	0-50, na 0-100
<i>C. tuberculata tuberculata</i> II	na <b>All types</b>	0-50 <b>1-1650</b>
<i>C. tuberculata tuberculata</i> III	Sand/Clay/Silt/Mud/na <b>All types</b>	51-500, >501, na <b>1-1650</b>
<i>C. tuberculata tuberculata</i> IV	Silt <b>All types</b>	0-50 <b>1-1650</b>
<i>C. tuberculata tuberculata</i> V	Sand <b>All types</b>	51-500 <b>1-1650</b>
<i>C. sinistrodentata</i> I	na <b>Silt/silted sand</b>	0-50 <b>5-1400</b>
<i>C. sinistrodentata</i> II	Silt/ <b>Mud</b> Silt/silted sand	0-50 <b>5-1400</b>
<i>C. excelsiformis</i>	Na <b>Sand</b>	0-50 20
<i>C. glomerata</i>	Sand	>501

	<b>Silted sands/Silt</b>	<b>20-1300</b>
<i>C. interposita</i>	Sand <b>All except silts</b>	0-50 5-150
<i>C. parva</i>	Mud <b>All types</b>	0-50 0-20
<i>C. pterygota</i>	Fine gravel/na	0-50

**Table S3: Results of evolutionary species delimitations with the 4 theta rule (Birky et al. 2010).**

Specimen numbers and species names refer to the 16S phylogeny (Figure 2).  $n^1$  = number of specimens for sister clade 1,  $n^2$  number of specimens for sister clade 2.  $\theta$  corresponds to the genetic distance within sister clades, being corrected for the number of specimens. D= genetic distance. na= non applicable - calculations could not be conducted because only one sequence (singleton) was present per phylogenetic sister clade. Ratios of D/ $\theta$  larger than 4 fulfil the criteria of the 4 theta rule and are indicated in bold.

Specimens of sister clades	Species	$n^1$ $n^2$	$\theta$ (within clades)	D (between clades)	Ratio D/ $\theta$
BL13, BL15; BK11	<i>C. golyschkiniae I</i> <i>C. golyschkiniae II</i>	2 1	0.007561 na	0.143	<b>18.877</b>
BL13, BL15; VB17, VB18	<i>C. golyschkiniae I</i> <i>C. golyschkiniae III</i>	2 2	0.007561 0.001599	0.175	<b>23.195</b>
BL13, BL15; VB14, VB16, VB15	<i>C. golyschkiniae I</i> <i>C. cf. golyschkiniae IV</i>	2 3	0.007561 0.000805	0.239	<b>31.575</b>
BK11; VB17, VB18	<i>C. golyschkiniae II</i> <i>C. golyschkiniae III</i>	1 2	na 0.001599	0.209	<b>130.841</b>
BK11; VB14, VB16, VB15	<i>C. golyschkiniae II</i> <i>C. cf. golyschkiniae IV</i>	1 3	na 0.000805	0.267	<b>331.196</b>
VB17, VB18; VB14, VB16, VB15	<i>C. golyschkiniae III</i> <i>C. cf. golyschkiniae IV</i>	2 3	0.001599 0.000805	0.255	<b>159.704</b>

BL12, BT27; BL10, BT22	<i>C. parallela I</i> <i>C. parallela II</i>	2 2	0.018877 0.008385	0.204	<b>27.044</b>
BL12, BT27; BT30, VPB9	<i>C. parallela I</i> <i>C. parallela III</i>	2 1	0.018877 0.019364	0.241	<b>31.892</b>
BL10, BT22; BT30, VPB9	<i>C. parallela II</i> <i>C. parallela III</i>	2 2	0.008385 0.019364	0.267	<b>35.348</b>
BT29, BT28 ; BK6	<i>C. sernovi insularis I</i> <i>C. sernovi insularis II</i>	2 1	0.002966 0.000000	0.145	<b>49.036</b>
BL27, BL28, VB31; VBP20	<i>C. lacustris baikalensis I</i> <i>C. lacustris baikalensis II</i>	3 1	0.000806 na	0.031	<b>38.609</b>
BL27, BL28, VB31; BL18, BK25, BK26, CL1, CL14	<i>C. lacustris baikalensis I</i> <i>C. lacustris baikalensis III</i>	1 5	0.000806 0.003745	0.056	<b>14.984</b>
VBP20; BL18, BK25, BK26, CL1, CL14	<i>C. lacustris baikalensis II</i> <i>C. lacustris baikalensis III</i>	1 5	na 0.003745	0.036	<b>9.668</b>
BK15; BK8	<i>C. tuberculata I</i> <i>C. tuberculata II</i>	1 1	na na	0.209	<b>na</b>
BK15; BT9	<i>C. tuberculata I</i> <i>C. tuberculata IIIa</i>	1 1	na na	0.157	<b>na</b>
BK15; BT9	<i>C. tuberculata I</i> <i>C. tuberculata IIIa</i>	1 1	na na	0.157	<b>na</b>
BK15; BT33, BT32, BT21	<i>C. tuberculata I</i> <i>C. tuberculata IIIb</i>	1 3	na 0.002385	0.179	<b>75.15</b>
BK15; BK19, BT10, BT14, BT7, BT11	<i>C. tuberculata I</i> <i>C. tuberculata IIIc</i>	1 5	na 0.000404	0.157	<b>na</b>
BK15;	<i>C. tuberculata I</i>	1	na	0.213	<b>na</b>



BK21	<i>C. tuberculata IV</i>	1	na		
BK15;	<i>C. tuberculata I</i>	1	na	0.219	<b>91.01</b>
BT18, BT20	<i>C. tuberculata V</i>	2	0.001583		
BK8;	<i>C. tuberculata II</i>	1	na	0.169	<b>na</b>
BT9	<i>C. tuberculata IIIa</i>	1	na		
BK8;	<i>C. tuberculata II</i>	1	na	0.169	<b>70.89</b>
BT33, BT32, BT21	<i>C. tuberculata IIIb</i>	3	0.002385		
BK8;	<i>C. tuberculata II</i>	1	na	0.169	<b>418.873</b>
BK19, BT10, BT14, BT7, BT11	<i>C. tuberculata IIIc</i>	5	0.000404		
BK8;	<i>C. tuberculata II</i>	1	na	0.225	<b>na</b>
BK21	<i>C. tuberculata IV</i>	1	na		
BK8;	<i>C. tuberculata II</i>	1	na	0.228	<b>94.07</b>
BT18, BT20	<i>C. tuberculata V</i>	2	0.001583		
BT9;	<i>C. tuberculata IIIa</i>	1	na	0.140	<b>na</b>
BK21	<i>C. tuberculata IV</i>	1	na		
BT33, BT32, BT21;	<i>C. tuberculata IIIb</i>	3	0.002385	0.146	<b>61.24</b>
BK21	<i>C. tuberculata IV</i>	1			
BK19, BT10, BT14, BT7, BT11;	<i>C. tuberculata IIIc</i>	5	0.000404	0.131	<b>324.06</b>
BK21	<i>C. tuberculata IV</i>	1	na		
BT9;	<i>C. tuberculata IIIa</i>	1	na	0.150	<b>93.76</b>
BT18, BT20	<i>C. tuberculata V</i>	2	0.001583		
BT33, BT32, BT21;	<i>C. tuberculata IIIb</i>	3	0.002385	0.154	<b>64.36</b>
BT18, BT20	<i>C. tuberculata V</i>	2	0.001583		
BK19, BT10, BT14, BT7, BT11;	<i>C. tuberculata IIIc</i>	5	0.000404	0.141	<b>88.44</b>
BT18, BT20	<i>C. tuberculata V</i>	2	0.001583		

BK21;	<i>C. tuberculata IV</i>	1	na	0.102	<b>64.09</b>
BT18, BT20	<i>C. tuberculata V</i>	2	0.001583		

1 **Cryptic diversity and speciation in endemic *Cytherissa* (Ostracoda, Crustacea) from**  
2 **Lake Baikal**

3

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25

26 **Abstract**

27 Lake Baikal (Siberia) is the most ancient and deepest of all ancient lakes on Earth. It holds a  
28 (mostly endemic) diversity of thousands of animal species, including a speciose radiation of  
29 ostracods of the genus *Cytherissa*. Applying molecular tools to this crustacean group reveals  
30 that several morphological species are actually species clusters. Based on combined 16S and  
31 28S DNA sequence data from thirteen classic *Cytherissa* species and one subspecies *sensu*  
32 Mazepova (1990), we recognize 26 different genetic *Cytherissa* species, 18 with  
33 morphological variation and eight truly cryptic species. These results suggest that the actual  
34 specific diversity of *Cytherissa* in Lake Baikal might easily be double of what is presently  
35 known.

36 Baikalian endemic species most likely live in the cradle in which they originated and this  
37 opens perspectives to infer modes of speciation. Our current distribution data of *Cytherissa*  
38 species provide first indications for both geographic (lakes basins and shores) and ecological  
39 (sediment type, water depth) separation. Our present data thus provide the first steps towards  
40 future, rigorous testing of focussed hypotheses on the causality of speciation through either  
41 allopatric isolation or parapatric ecological clines.

42 **Keywords:** allopatric speciation, parapatric speciation, depth distribution, sediment types,  
43 lake basins, east-west shores, sexual reproduction

44

45

## 46 **Introduction**

47 Ancient lakes are natural laboratories for evolutionary studies on the tempo and mode of  
48 speciation of their endemic fauna and flora (Martens, 1997). There are only a couple of  
49 dozens of these lakes on the globe and they are hotspots of biodiversity, because of their high  
50 endemicity and their importance for generating diversity in surrounding areas (Schön &  
51 Martens, 2012). Non-marine ostracods are not only an important ecological component of  
52 ancient lake taxa, but ancient lakes also contribute up to 25% of all known non-marine  
53 specific ostracod diversity (Martens et al., 2008).

54 On a global scale, Lake Baikal is the oldest extant lake with an estimated age of 25-30 myr  
55 (Sherbakov, 1999; Müller et al., 2001) as well as the deepest lake with a maximal depth of  
56 more than 1600 m (Sherstyankin et al., 2006). About 2500 animal (morpho) species occur in  
57 Lake Baikal, of which 1455 are endemic (Timoshkin, 2001). Concerning non-marine  
58 Ostracoda, more than 90% of Baikalian ostracods are endemic to the lake, and the *Cytherissa*  
59 species flock has the highest specific diversity (Mazepova, 1990). This species flock is  
60 probably an example of explosive radiation and has originated 5-8 million years ago (Schön  
61 & Martens, 2012), at a time when Lake Baikal's cold, oxygenated abyss was formed  
62 (Sherbakov, 1999).

63 With the availability of molecular tools, the last twenty years have seen an ever-increasing  
64 number of studies detecting so-called cryptic diversity (Bickford et al., 2007), i.e. lineages  
65 that are morphologically similar but fulfil all criteria to be different genetic species (Vogler &  
66 Monaghan, 2007) according to the phylogenetic species concept (Eldredge & Cracraft  
67 (1980), but see also overview in Zhang et al., 2013) or the evolutionary genetic species  
68 concept (Birky & Barraclough, 2009). There is mounting evidence that cryptic species occur  
69 widely and that their presence is, at least in part, linked to specific types of habitat. For

70 example, freshwater taxa show significantly more cryptic diversity than either terrestrial or  
71 marine taxa (Poulin & Pérez-Ponce de León, 2017).

72 Also in non-marine ostracods, cryptic species have been detected, varying between eight in a  
73 putative ancient asexual darwinulid species (Schön et al., 2012) to more than 35 in a single  
74 Holarctic temporary pool species (Bode et al., 2010). Likewise, cryptic species have been  
75 found in endemic *Romecytheridea* ostracods from Lake Tanganyika, the second most ancient  
76 lake in the world (Schön et al., 2014). The discovery of cryptic lineages throughout all  
77 metazoan phyla (Beheregaray & Caccone, 2007; Pfenninger & Schwenk, 2007) is not only  
78 important for fundamental science and systematics, but has also profound implications for  
79 conservation and management (examples in Brown et al., 2007; Elmer et al., 2007; Fontaneto  
80 et al., 2008; Gustafsson et al., 2009; Marrone et al., 2010), especially in unique environments  
81 such as ancient lakes. Indeed, if genetic diversity is cryptic, it is equally difficult to recognize  
82 it and to protect it from extinction.

83 Here, we use mitochondrial and nuclear DNA sequence data to test for the presence of cryptic  
84 species within 14 known morphological *Cytherissa* species and subspecies. Our samples  
85 come from all three basins of Lake Baikal, from both eastern and western shores and from  
86 both different water depths and different types of sediments, enabling us to assess the recent  
87 distribution patterns of these ostracods. Our research provides preliminary indications on the  
88 causal importance of allopatric isolation (different basins or shores) and parapatric ecological  
89 speciation (depth, sediment types) for the past radiation of endemic *Cytherissa* species in  
90 Lake Baikal.

91

## 92 **Material and Methods**

93 *Sampling*

94 During four expeditions on Lake Baikal, in 1999, 2007, 2009 and 2011, several dozens of  
95 samples for ostracods were collected by SCUBA diving, trawling, dredging and with the  
96 oceanographic Reineck box-corer, from various locations in Lake Baikal, including both the  
97 eastern and western shore and all three basins and at depths as from 20m in the littoral photic  
98 zone (0–100 m) to deep water habitats of more than 500 m. Ostracods were sorted alive under  
99 a light microscope on the research vessels, were fixed in cold 95% pure ethanol for  
100 subsequent analyses and separated into preliminary taxonomic groups using the valve  
101 outlines of Mazepova (1990) and the hemipenis outlines by Van Mulken et al. (in prep.). We  
102 also sampled *Cytherissa lacustris*, the recent extra-lacustrine spin-off of the Baikalian  
103 *Cytherissa* flock (Schön & Martens, 2012), from Semerwater in the UK (see Table 1 for more  
104 details).

105

106 *DNA extraction, PCR and sequencing*

107 For most specimens, valves were removed for Scanning Electron Microscopy (SEM) and the  
108 remaining soft parts were used to extract DNA from individual ostracods with a slightly  
109 modified protocol of the DNA Easy Blood and Tissue kit (Qiagen), adjusting the elution  
110 volumes because of the small size of individual ostracods. We estimated the concentration of  
111 the obtained DNA extractions with the Nanodrop and used the eluate with the highest  
112 concentration for all subsequent steps of the molecular analysis. With PCR (Polymerase  
113 Chain Reaction), we amplified part of the mitochondrial 16S ribosomal region with specific  
114 primers (16S-F3 TTAATTCAACATCGAGGTCACAA and 16S-R2  
115 GAGTAAACGGCTGCAGTA) and the D1D2 part of the nuclear Large Subunit (28S) with  
116 universal primers (D1D2Fw1 5'-AGCGGAGGAAAAGAACTA-3') and (D1D2Rev1 5'-



117 TACTAGAAGGTTTCGATTAGTC-3') (Sonnenberg et al., 2007). Both regions have been  
118 successfully sequenced in other studies on non-marine ostracods (Bode et al., 2010; Koenders  
119 et al., 2012; Schön et al., 2014), and have also been used for the detection of cryptic species.  
120 PCRs were conducted in a T personal Thermoblock (Biometra) with 25 µl volumes of the  
121 Qiagen HotStar Mastermix (1.5 mM MgCl<sub>2</sub>, 200µM dNTP, Tris·Cl, KCl, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.25 U  
122 Taq), 0.1 µM of each primer and the following cycling conditions: 15 min at 95°C, followed  
123 by 40-42 cycles of 1 min at 95°C, 1min at 44°C (16S) and 48°C (28S) and 1 min at 72°C,  
124 followed by a final extension step of 72°C for 10 minutes. We used agarose gel  
125 electrophoresis and stained gels with GelRed to check if PCR amplifications were successful.  
126 Positive amplicons were purified with the GFX PCR DNA and gel band purification kit (GE  
127 Healthcare) kit and sequenced in both directions using the PCR primers and the Big Dye kit  
128 (ABI) on an ABI 3130x1 capillary DNA sequencer (Life Technologies).

129

### 130 *Analyses of DNA sequence data*

131 We visualized sequencing chromatograms and generated consensus sequences for each  
132 specimen with Bioedit (Hall, 1999). Sequence ambiguities were checked by eye and  
133 corrected manually, sequences were aligned with MAFFT (Kato & Standley, 2013) on  
134 <http://www.ebi.ac.uk> and trimmed to equal lengths in BioEdit. Sequence identity was  
135 confirmed by BLAST searches in Genbank (Altschul et al., 1997). As outgroup, we used  
136 sequence data from *Romecytheridea ampla*, an ostracod species from Lake Tanganyika  
137 belonging to the same subfamily and from which both 16S and 28S sequence data were  
138 available (Table 1). We also combined the DNA sequence data from both markers into a  
139 congruent dataset with Sequence Matrix (Vaidya et al., 2011). We trimmed the final  
140 alignment for each dataset with the outgroup (Table 1) to equal length and selected the best-

141 fitting evolutionary model in jModeltest 2 (Darriba et al., 2012) using model filtering, the  
142 corrected Akaike Information Criterion (AICc) and 88 different nucleotide substitution  
143 models. The parameters of the best-fitting evolutionary models were used in phylogenetic  
144 reconstructions with Maximum Likelihood (ML) (PHYML; Guindon & Gascuel, 2003) and  
145 Bayesian approaches (MrBayes 3.2; Ronquist et al., 2012). Not all models selected by  
146 jmodeltest2 are implemented in MrBayes and we therefore had to pick the closest ones for  
147 Bayesian analyses. For 16S, the TIM1+I+G model was selected with freqA = 0.2927; freqC =  
148 0.2296; freqG = 0.1968; freqT = 0.2810; [AC] = 1.0000; [AG] = 5.4559; [AT] = 0.6273;  
149 [CG] = 0.6273; [CT] = 3.1987; [GT] = 1.0000; p-inv = 0.2280; gamma shape = 0.4750.  
150 For 28S, the TPM3uf+ G model was selected with the following parameters: freqA = 0.1981;  
151 freqC = 0.2286; freqG = 0.3059; freqT = 0.2675; [AC] = 0.0000; [AG] = 3.6775; [AT] =  
152 1.0000; [CG] = 0.0000; [CT] = 3.6775; [GT] = 1.0000; gamma shape = 0.3010. For the  
153 combined dataset, the TIM1+I+G model was selected with freqA = 0.2632; freqC = 0.2145;  
154 freqG = 0.2316; freqT = 0.2907; [AC] = 1.0000; [AG] = 5.3221; [AT] = 0.5999; [CG] =  
155 0.5999; [CT] = 3.5948; [GT] = 1.0000; p-inv = 0.4740; gamma shape = 0.3080. In all cases,  
156 we constructed ML trees in PHYML with these parameters and 1000 bootstraps. We also  
157 constructed ML trees without bootstrap support and outgroups and from haplotype  
158 sequencing only for the Poisson Tree Processes (PTP) algorithm (Zhang et al., 2013) to  
159 delimitate genetic species (see below). For Bayesian approaches, we ran MrBayes with two  
160 MCMC chains and 20 million generations, applying the GTR+I+G model for 16S and the  
161 combined dataset and the HKY85+G model for 28S, and sampling trees every 1000  
162 generations. After inspecting the results, we eliminated the first 20000 trees as burn-in and  
163 calculated the 50% majority rule consensus tree. All trees were visualized and manipulated  
164 with MEGA 6.0 (Tamura et al., 2013) and FigTree (Rambaut, 2017).

165

166 *Networks*

167 To obtain the best graphic representation of haplotypes and their connectivity at the  
168 population level, we also constructed minimum spanning (Bandelt et al., 1999) networks  
169 from the 16S and 28S data with popart 1.2 (<http://popart.otago.ac.nz>) colour-coding the  
170 geographic origin (lake basin) as traits.

171

172 *Delimitating genetic species*

173 We used two different methods for quantitative delimitations of genetic species based on the  
174 evolutionary genetic species concept (Birky & Barraclough, 2009), nl. the 4  $\theta$  (theta) rule  
175 (Birky et al., 2010; Birky, 2013) and the PTP algorithm (Zhang et al., 2013). For applying the  
176 4  $\theta$  rule, we first identified well-supported phylogenetic sister clades from the ML and  
177 Bayesian phylogenies with a bootstrap support of more than 75% or a posterior probability of  
178 more than 0.8. Within and between the sister clades, we then calculated genetic distances in  
179 MEGA 6.0 using the appropriate model for molecular evolution. As with Bayesian analyses,  
180 not all models selected by jmodeltest2 are available in MEGA and we chose the closest ones  
181 for the calculation of genetic distances. Next,  $\pi$  (nucleotide diversity) and  $\theta$  (population  
182 mutation rate) were calculated taking sampling size of each sister clade into account. Finally,  
183 we calculated D (distance between sister clades) and the ratio between  $\theta$  and D. If the  
184 resulting ratio is greater than 4, sister clades are considered to be different evolutionary  
185 species (Birky et al., 2010).

186 We also used a Poisson Tree Processes (PTP) model to delimit genetic species. This  
187 algorithm is based on a shift of the Poisson distributions of substitution rates of branches  
188 within and between species in a phylogenetic tree (Zhang et al., 2013) The ML trees of 16S,  
189 28S and the combined 16S/28S dataset were uploaded on the website of bPTP (<http://sco.h-its.org/exelixis/web/software/PTP>) without outgroups and bootstraps and only representing

191 individual haplotypes . The statistical support of potential genetic species was calculated with  
192 the maximal possible number of 500,000 MCM generations and the default burn-in of 10%.  
193 For comparisons, we also applied a third approach for genetic species delimitations, the  
194 Automatic Barcode Gap Discovery method (ABGD; Puillandre et al., 2012) which calculates  
195 genetic distances between all sequences ~~and does. It does~~ not require any phylogenetic  
196 information. ~~but it has the weakness that it is not based on underlying theoretical content~~  
197 ~~(Hull, 1997). We did not use a fourth method commonly used for genetic species~~  
198 ~~delimitations, the GMYC algorithm (Pons et al., 2006), because this method requires dated,~~  
199 ~~ultrametric trees. However, thus far no suitable molecular clocks are available for 16S or 28S~~  
200 ~~sequences from non-marine ostracods.~~

201

#### 202 *Statistical analyses of current distribution data*

203 We summarize current distribution data of all genetic species defined by the congruent  
204 molecular data sets regarding ecological (sediment type, water depth), and geographic  
205 (different lake basins, different shores) factors. We also compare our ecological distribution  
206 data to the much larger dataset of Mazepova (1998) on different sediment types and water  
207 depths of morphological *Cytherissa* species and subspecies.

208 We then generated a presence-absence matrix for each genetic species from the combined  
209 molecular dataset for the four distribution variables, using our geographic and ecological data  
210 and the ecological data of Mazepova (1998). This data matrix was used for ordination  
211 analyses in PAST (Hammer et al., 2001). More specifically, we conducted a Principal  
212 ~~Coordination~~ ~~Analysis~~ Analysis (PCoA) with the jaccard similarity index, and the default  
213 transformation exponent of 2. This kind of analyses plots the distribution of genetic  
214 *Cytherissa* species in a coordination system where the axes are linked to the different  
215 distribution variables.

216

## 217 **Results**

218

### 219 *DNA extraction*

220 We have extracted DNA from more than 100 specimens, and have been successful in  
221 obtaining 68 sequences for 16S and 83 sequences of 28S, respectively (Table 1). Developing  
222 suitable primers for 16S has been a major obstacle and has involved several rounds of  
223 redesigning both forward and reverse primers. Problems with the primers are also the reason  
224 why we could not successfully follow the approach of Schön & Martens (2012) in acquiring  
225 more COI sequences from the same species and localities, which would have been very  
226 useful for further comparisons. Also, the specimens or DNA extractions of Schön & Martens  
227 (2012) were no longer available to be included in the current study.

228

### 229 *Molecular taxonomy*

#### 230 *Combined molecular datasets*

231 Combining both molecular datasets has resulted in phylogenetic trees with some higher  
232 support for deeper nodes in the upper part of the tree (Figure 2) than the phylogenies that  
233 were based only on 16S (Figure S1) or 28S (Figure S2). The terminal branches in Figure 2  
234 are generally well supported with bootstrap values of 75% or more and posterior probabilities  
235 of more than 0.8. In the combined 16S/28S tree, such well-supported clades consist of sister  
236 groups (*C. lacustris* I and II; *C. golyschkiniae* I and II; *C. parallela* I and II) but also of  
237 clusters of different morphological (sub)species (*C. parva* and *C. sp. 3*; *C. parallela* III and  
238 both *C. lacustris* I and II; *C. sernovi insularis* I and *C. sernovi sernovi*). The remaining part

239 of the tree, however, still contains many polytomies, especially at the deeper nodes. With the  
240 exception of *C. lata* I and II and *C. tuberculata tuberculata* IV and V, respectively, the  
241 phylogenetic relationships of the eight other clades remains unresolved.

242 The 16S and 28S DNA sequences come from 13 known morphological species and one  
243 subspecies *sensu* Mazepova (1990) plus four new species that await formal description  
244 elsewhere. With the combined molecular data, we identified 26 well-supported phylogenetic  
245 clades (Figure 2). Many of these are congruent with morphological species (*C. parva*, *C.*  
246 *pterygota*, *C. interposita*, *C. excelsiformis*, *C. glomerata* and four yet undescribed species (*C.*  
247 *spec.* 1 to 4) plus one subspecies (*C. sernovi sernovi*). There are an additional five  
248 morphospecies with multiple, well-supported phylogenetic clades or with phylogenetically  
249 distant sister clades, both indicating possible cryptic species. *Cytherissa tuberculata*  
250 *tuberculata* splits into four such clades and *C. parallela* into three, while two each are found  
251 in *C. lacustris*, *C. sernovi insularis*, *C. golyschkiniae*, *C. sinistrodentata*, and *C. lata* (Figure  
252 2).

253 We have used two different methods to test if these phylogenetic clades fulfil the criteria to  
254 be considered different evolutionary genetic species. Because of the more limited number of  
255 specimens for which DNA sequence data are available from both genomic regions, the  
256 number of singletons in the congruent phylogeny (Figure 2) is larger than in the 16S tree  
257 (Figure S1). Singletons cause potential problems when applying the 4  $\theta$  rule (see below).  
258 Still, this method supports 17 genetic species within morphospecies (Table 2, Table S1) plus  
259 another eight morphospecies (Figure 2). The PTP algorithm recognizes all of the clades from  
260 the 4  $\theta$  rule (Table 2) and additionally splits *C. parallela* I and II into two different genetic  
261 species with one singleton each (Table 2). The ABDG method delimitates the same species as  
262 PTP ([when using the 16S data for ABDG](#); not shown, data are available from IS on request).  
263 We take a conservative approach in delimitating species, using support from all three

264 methods and therefore regard the two clades of *C. parallela* I and II for now as two genetic  
265 species. We can then recognize a total of 26 different genetic species from the combined  
266 molecular data. For most of these genetic species, we find variation in valve characters  
267 (indicated in bold in Figure 2), thus providing morphological support for genetic species  
268 boundaries. Many of these species are also the same as in Mazepova (1990). We also found  
269 four new morphological species (*C. sp.* 1 to 4) that are, with the exception of *C. sp.* 1, also  
270 fully supported by the combined molecular data. Other genetic species resemble the species  
271 *sensu* Mazepova (1990) to some extent but show additional valve differences. These species  
272 are still awaiting a formal taxonomic description and are for now indicated with Roman  
273 numbers after the original species name in Figure 2 (*C. parallela* I-III and *C. tuberculata*  
274 *tuberculata* II-V - see Fig S3, SEM plate). However, in the other instances of genetic species  
275 with Roman numbers in Figure 2, no clear morphological differences are found and these  
276 eight remaining lineages are here considered to be true cryptic species.

277

#### 278 *16S results*

279 Because we could obtain more sequence data from this marker than could be used for the  
280 combined data set, numbers of genetic species are slightly different. If we apply the PTP  
281 algorithm or the ABDG method (not shown, data are available from IS on request), we can  
282 identify 35 genetic species (Figure S1). With the 4  $\theta$  rule, the result is, with 36 genetic  
283 species, rather similar, but individual species delimitations are incongruent for morphospecies  
284 with several genetic species (Table S3).

285 When comparing the 16S species boundaries to morphological variability as we did for the  
286 combined molecular data set, we find a total of nine truly cryptic species in the 16S dataset  
287 (Figure S1), one more than with the 16S/28S data.

288 The structure of the 16S minimum spanning network (Figure S4) matches the well-supported  
289 phylogenetic clades in Figure S1. We find 58 different haplotypes that are separated from  
290 each other by more than 20 mutational steps. Within evolutionary genetic species such as for  
291 example *C. tuberculata tuberculata* III, *C. lacustris* II or *C. golyschkiniae* IV, we also find  
292 haplotypes differing only by small numbers of mutational steps.

293

#### 294 *28S results*

295 The nuclear ribosomal 28S region shows very little genetic variability amongst Baikalian  
296 *Cytherissa* species. Consequently, the phylogenetic tree is unresolved with very few  
297 exceptions (see Figure S2). There are only 18 haplotypes in the minimum spanning network,  
298 with a maximum of four mutation steps (Figure S5) although more than 80 specimens from  
299 have been sequenced from the entire lake. The network shows three very common 28S  
300 haplotypes (Figure S5). The most frequent one is present in more than ten different  
301 morphospecies and subspecies. Except for one specimen of *C. golyschkiniae* and *C. verrucosa*  
302 each that are separated by four mutation steps from the next haplotype (Figure S5), all other  
303 single 28S haplotypes are only one or two mutational steps away from the three most  
304 common haplotypes or from each other. Because of the limited genetic diversity of 28S, we  
305 did not use these DNA sequence data to delimitate genetic species boundaries.

306

#### 307 *Current distribution of genetic Cytherissa species*

308 Our sampling scheme contains habitats with different ecological (sediment type, water depth)  
309 and geographic features (south, central and northern basin; and east and west shores), which  
310 could have contributed to different distributions of the genetic *Cytherissa* species. Because



311 our sample numbers are somewhat limited, we have compared our distribution data to the  
312 larger dataset of Mazepova (1998; Table S2). It seems that certain morphological  
313 (sub)species have previously been found on more sediment types than in our study (e. g. *C.*  
314 *golyschkiniae*, *C. tuberculata tuberculata*, *C. excelsiformis* and *C. glomerata*; Table S2).  
315 Mazepova (1998) also reported a wider depth distribution for these three morphological  
316 (sub)species as well as for *C. sinistrodentata*. For the remaining seven genetic species, our  
317 data match the depth distributions of Mazepova (1998) well.

318 Table 3 summarize the distribution data of all genetic species from the congruent molecular  
319 dataset, arranged in pairs of genetic sister clades to allow easy comparisons. A PCoA analysis  
320 of these data shows that most genetic species are well separated from each other (Figure 3),  
321 also the species pairs from Table 3 and the various cryptic species (see above). The first axis  
322 with an eigenvalue of 1.5139 explains 35.639% of the overall variation and the second one  
323 22.0325 %, which are relatively high scores.

324

## 325 **Discussion**

### 326 *Phylogenetic and network structures*

327 We have sequenced two different genetic markers, namely part of the mitochondrial 16S and  
328 part of the nuclear 28S ribosomal region from 18 morphological (sub)species of *Cytherissa*.  
329 The molecular phylogenies from both genomic regions show many polytomies, especially of  
330 the deeper nodes, regardless of the methods used for phylogenetic reconstructions (Figure 2,  
331 S1 & S2) and when using the two datasets either separately or combined (Figures 2, S1-S2).  
332 In our 16S and combined 16S/28S trees, only the terminal nodes and some deeper nodes  
333 (16S/28S) are statistically well supported (Figure 2) whereas almost the entire 28S phylogeny  
334 remains unresolved (Figure S2). Our phylogenetic results thus resemble those of Schön &  
335 Martens (2012), as also in their study, the mitochondrial phylogeny of Baikalian *Cytherissa*  
336 ostracods based on COI had many polytomies, and only the terminal nodes were well  
337 supported in the mitochondrial gene (COI), while the nuclear phylogeny (from the ITS1  
338 region) was not resolved at all. Similar incongruences in genetic variability between  
339 mitochondrial and nuclear markers have also been reported from other studies on ostracods  
340 (Schön et al., 1998, 2010, 2012, 2014; Brandao et al., 2010; Koenders et al., 2012), and on  
341 meiofauna in general (Tang et al., 2012), resulting in low phylogenetic resolution and  
342 polytomies. One potential causality for this discrepancy is that nuclear ribosomal regions in  
343 non-marine ostracods generally seem to evolve at a much slower pace than mitochondrial  
344 regions (Schön et al., 2003). Even more relevant here is the detection of explosive speciation  
345 in Baikalian *Cytherissa* (Schön & Martens, 2012), which explains best why our phylogenies  
346 are unresolved at the base of the trees.

347 The topology of our combined tree (Figure 2) reveals certain similarities with the trees in  
348 Schön and Martens (2012), as *C. tuberculata* is closest to the root of the *Cytherissa* flock and

349 *C. parva* forms a well-supported clade with *C. sp. 3*, apart from the other *Cytherissa* species.  
350 However, in our present results we cannot detect the four well-supported clades from the COI  
351 tree of Schön & Martens (2012). These inconsistencies could be owing to differences in  
352 genetic variability between 16S and COI and the fact that our dataset is not fully congruent  
353 with the data of Schön & Martens (2012). Because these authors used other specimens, it is  
354 also not possible to combine and re-analyse all existing molecular data of *Cytherissa*. The  
355 problem of resolving the Baikalian *Cytherissa* phylogenies urgently calls for the development  
356 of more suitable, large scale molecular markers such as sequencing entire mitogenomes  
357 (Schön & Martens, 2016) or large scale genomic data from multiple markers, such as those  
358 Meyer et al. (2015) developed for cichlid fish.

359

### 360 ***Diversity of Baikalian Cytherissa***

361 Our results show that the actual biodiversity of endemic *Cytherissa* in Lake Baikal is higher  
362 than previously thought. Mazepova (1990) recognized a total of 47 species and 10 subspecies  
363 of *Cytherissa* based on valve characters. We have used SEM (see above and Fig S3) to study  
364 differentiation of valve morphology in all specimens and also characterised hemipenis  
365 morphology for selected *Cytherissa* species (Van Mulken et al. in prep.). Both methods  
366 provide a much finer resolution of morphological differentiation, as is illustrated by the four  
367 (16S/28S combined) to five (16S) new *Cytherissa* species that we found and that are  
368 confirmed with our genetic data. Also, in three *Cytherissa* morphospecies *sensu* Mazepova  
369 (1990), we can distinguish nine genetic *Cytherissa* species with clear differences in valve  
370 morphologies, that also fulfil the criteria of the evolutionary genetic species concept using the  
371 combined DNA sequence data (*C. parallela* I-III, *C. lacustris* I & II, *C. tuberculata*  
372 *tuberculata* II-V; see Figure S3 for the latter).

373 We have furthermore detected several truly cryptic species (without any apparent  
374 morphological differentiation), which supports the first indications reported by Schön &  
375 Martens (2012) for cryptic speciation in Baikalian ostracods. Using the combined molecular  
376 data set, we identify eight cryptic species (nine when only using the 16S; see Figure S1).  
377 When applying two different statistical methods to delimitate evolutionary genetic species,  
378 the 4  $\theta$  rule and the PTP algorithm, the overall estimate was for the combined dataset  
379 relatively similar with 26 and 27 species, respectively.

380 But our combined 16S/28S tree has many well supported clades with singletons (Figure 2),  
381 and the genetic diversity within such clades is zero. Consequently, the ratios would have to  
382 be divided by zero and can thus not be calculated (Table S1). The results of the PTP  
383 algorithm are probably more robust as this method can also be used for singletons and we  
384 found furthermore the same genetic species when applying the ABDG method and this for  
385 both 16S and the combined molecular dataset. We could not increase the number of  
386 specimens in our study, because of the difficulties to obtain more 16S sequences (see above)  
387 and because of low densities of non-marine ostracods in Lake Baikal, especially at greater  
388 depths.

389 In total, we can identify 26 different genetic species with the combined molecular data set,  
390 representing 14 morphological (sub)species *sensu* Mazepova (1990). Our data thus almost  
391 double the previously known diversity of endemic *Cytherissa* species from Lake Baikal. Our  
392 sampling includes all three basins of Lake Baikal, five different sediment types and water  
393 depths ranging from shallow habitats (c 20 m) to more than 500m, covering most of the  
394 habitat and geographical diversity of Lake Baikal. Extrapolating our results on morphological  
395 and cryptic diversity to the entire Baikalian *Cytherissa* species flock (26 genetic species in 14  
396 morphospecies *sensu* Mazepova (1990)) implies that we can expect almost twice as many  
397 (cryptic) *Cytherissa* species from Lake Baikal as previously known, with therefore one

398 hundred species, including cryptic ones, being a more realistic estimate than the 47  
399 morphological (sub)species *sensu* Mazepova (1990) previously known. Studies on ostracods  
400 from other ancient lakes have also reported the presence of cryptic species, thus considerably  
401 increasing classic diversity estimates (Schön et al., 2014; Karanovic, 2015). Likewise, cryptic  
402 species have also been found in Baikalian amphipods (Vainola & Kamaltynov, 1999) and in  
403 Baikalian sponges (Itskovich et al., 2015).

404 High cryptic diversity and cryptic speciation in ancient lakes somewhat negates the recent  
405 findings by Poulin & Pérez-Ponce de León (2017), who attributed the higher cryptic diversity  
406 in freshwater as compared to terrestrial and marine habitats, to the greater heterogeneity of  
407 freshwater habitats. This hypothesis mostly refers to the patchiness and isolation of the many  
408 freshwater pools, lakes and rivers. However, in the case of ancient lakes, their long  
409 evolutionary history, large size and unusual depth are probably more important for generating  
410 cryptic diversity than providing many heterogeneous habitats.

411 Based on classic morphological species boundaries, ancient lakes have already been  
412 identified as major hotspots for non-marine ostracod diversity as, for example, they  
413 contribute 25% of all known freshwater ostracod species (Martens et al., 2008). The increase  
414 of the known diversity through the discovery of cryptic species from our and other studies  
415 emphasises the importance of ancient lakes as biodiversity hot spots, not only for ostracods.  
416 This has major implications for the conservation and protection of these lakes and their  
417 unique fauna and flora, even outside the lakes themselves (Schön et al., 2000; Schön &  
418 Martens, 2012)

419

420 ***Factors linked to speciation in Baikalian Cytherissa***

421 Ancient lakes are *in situ* laboratories for evolutionary studies in general, and to investigate  
422 the factors that have promoted and caused speciation, giving rise to the impressive endemic  
423 diversity of these lakes in particular. Mayr (1942, 1963) regarded geographic isolation as the  
424 most important driver for (allopatric) speciation and this view dominated the field for a long  
425 time. Meanwhile, also the importance of intrinsic factors for sympatric speciation in ancient  
426 lakes has been recognized (see, for example, Schön & Martens, 2004 and Cristescu et al.,  
427 2010), with cichlid fish still being the most prominent example (e. g. Muschick et al., 2012).  
428 Martens (1994, 1997) furthermore re-iterated the term “parapatric speciation”, describing  
429 isolation and gene flow along an ecological or geographical gradient, which is highly  
430 applicable to Lake Baikal with its deep, fully oxygenated abyss (down to 1600 m), and its  
431 north-south length of more than 600 km (Martin, 1994). Because our study detected at least  
432 26 genetic species of *Cytherissa*, including some cryptic species, and because our sample  
433 scheme included all three basins of Lake Baikal from the eastern and western shore, depths  
434 ranging from shallow to deep water habitats and five sediment types, we can make a first  
435 attempt to assess how recent *Cytherissa* species could be ecologically and geographically  
436 separated. Because of the limited number of molecular data currently available and the lack  
437 of extensive, dated phylogenies, our analyses can only provide the very first steps towards  
438 future, rigorous testing of hypotheses on allo- or parapatric speciation of non-marine  
439 ostracods in Lake Baikal in general and of selected *Cytherissa* clades in particular.

440

#### 441 *Geographic and ecological separation*

442 Geographic separation because of historical vicariance might to some extent have shaped  
443 *Cytherissa* diversity, and possibly, also speciation in Lake Baikal. Our PCoA illustrates that  
444 most genetic *Cytherissa* species are clearly separated by extrinsic factors (Figure 3), even if  
445 we use the (wider) ecological distribution data of Mazepova (1998) for water depths and

446 sediment for morphological species and subspecies without being able to differentiate further  
447 according to our genetic species (see Table S2). What is more difficult to assess is the extent  
448 to which each factor might have contributed to the current ecological and geographic  
449 distribution and to speciation in the past. We find several examples where different (cryptic)  
450 *Cytherissa* species seem to be limited in their geographic distribution to a single Baikalian  
451 basin or shore (species pairs: *C. lacustris* I & II; *Cytherissa sernovi insularis* II & *C. sernovi*  
452 *sernovi*, *C. lata* I & II, *C. tuberculata tuberculata* IV & V; see Table 3). Distribution patterns  
453 potentially resulting from allopatric speciation amongst basins have also been described from  
454 Lake Tanganyika for ostracods (Schön et al., 2014) and cichlid fish (Snoeks et al., 1994;  
455 Rüber et al., 1999, 2001; Sturmbauer et al., 2001; Nevado et al., 2009, 2011). Keeping the  
456 lack of ancestral reconstructions and thus rigorous testing of this hypothesis in mind, our  
457 preliminary indications for allopatric speciation in Lake Baikal are still noteworthy as only  
458 one other case of supposed allopatric speciation from this lake is documented up to now,  
459 namely the case of *Eulimnogammarus cyaneus* versus *E. messerschmidtii* (Bedulina et al.,  
460 2014).

461 Other examples in our dataset show that besides geographic separation, also ecological  
462 factors like water depths or sediment types might have further contributed to the current  
463 disjunct distribution of certain *Cytherissa* species (e.g. the species pairs *C. parallela* I & II;  
464 *C. lata* I & II and *Cytherissa tuberculata tuberculata* IV & V, see Table 3).

465 For most of the genetic *Cytherissa* species, our depth ranges match the ones of Mazepova  
466 (1998) remarkably well. Exceptions include *C. glomerata* but also the common  
467 morphospecies *C. sernovi*, *C. sinistrodentata* and *C. tuberculata tuberculata*, for which  
468 Mazepova (1998) reported much wider depth distributions than we found for our different  
469 genetic species constituting these classic morphospecies (Table S2). Whether or not there is  
470 indeed a clear separation between the genetic/ cryptic species by water depths and/or by

471 sediment type (as in snails from Lake Tanganyika; Michel et al., 1992) still has to be further  
472 tested with more extensive sampling and subsequent genetic characterisation.

473

#### 474 *Potential intrinsic factors*

475 We can currently not assess at all to which extent intrinsic factors or adaptive evolution have  
476 caused sympatric ostracod speciation. To investigate trophic niches, for example, detailed  
477 morphological investigations of the appendages involved in food processing or stable isotope  
478 analyses would be needed. Preliminary analyses of soft part morphology in *Cytherissa* offers  
479 no indication for trophic specialisation in the relevant head appendages (Danielopol & Tétart,  
480 1990).

481 To study other intrinsic mechanisms, like for example hybridisation or introgression which  
482 are common in cichlids from African ancient lakes (Koblmüller et al., 2007; Nevado et al.,  
483 2009, 2011; Cristescu et al., 2010; Genner & Turner, 2011; Anseeuw et al., 2012; Meier et  
484 al., 2017), is not possible to date as no suitable molecular tools are as yet available for  
485 ostracods. It remains therefore uncertain whether the large number of 28S haplotypes shared  
486 between different *Cytherissa* species (Figure S5) is a first indication for hybridisation or is  
487 merely a reflection of the low variability of this nuclear region.

488 Sexual selection has often been cited as a major driver in ancient lake speciation, and the best  
489 documented examples are of course (again) the cichlid fish (reviewed in Wagner et al. 2012).

490 Sexual selection in ostracods has previously been documented in both freshwater (Martens,  
491 2000) and marine groups (Tsukagoshi, 1988), and is often detectable by wide morphological  
492 differences in copulatory structures (hemipenes, prehensile palps) between otherwise closely  
493 related species. However, the study by Van Mulken et al. (in prep.) shows that the copulatory  
494 appendages in Baikalian *Cytherissa* species, albeit quite elaborate, are very similar amongst  
495 otherwise different species.



496 *Cytherissa lacustris* II is found in both Lake Baikal and in the UK (Table 1; Figure S4; Schön  
497 & Martens, 2012) and has different reproductive modes. In Lake Baikal, its “sors” (shallow  
498 lagunas associated with the lake) and in Lake Huvsugul (Mazepova, 2006) it is fully sexual,  
499 while in the rest of the Holarctic it is obligate asexual (Schön et al., 2000; Schön & Martens,  
500 2012). This variation in reproductive mode indicates that such intrinsic factors might also be  
501 relevant for ostracod speciation in ancient lakes (Martens, 1994) and elsewhere, and need to  
502 be studied with suitable tools.

503

## 504 **Conclusions**

505 To summarize, we found strong evidence from two molecular markers and morphological  
506 variation that the *Cytherissa* diversity from Lake Baikal is probably e-twice as large as  
507 previously known. Our preliminary data also indicate that the 26 genetic species are to some  
508 extent separated by ecological (sediment types, water depths) as well as geographic (basin,  
509 shore) factors. We argue that these separations might have been causal to allopatric and  
510 parapatric speciation along gradients without complete isolation. In the case of Lake Baikal,  
511 such gradients include the vast geographic distances among the three basins, between the two  
512 shores (east and west) and the large ecological gradient in water depth down to c 1600 m.  
513 These hypotheses will need to be rigorously tested in future research.

514 Other external factors possibly promoting speciation, such as multiple invasions, have  
515 already been documented (Schön & Martens, 2012), while also adaptive and intrinsic  
516 components are expected to have further contributed to generating the high diversity of  
517 Baikalian ostracods and other endemic taxa. We hope that with the increasing availability of  
518 various “omics” techniques, also applicable to ostracods (Schön & Martens, 2016), future  
519 studies will be able to answer these fundamental questions of evolutionary biology, and in  
520 particular on speciation in ancient lakes.

521

522

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748

749

750 **Captions of Figures and Tables**

751 **Tables:**

752 **Table 1: Overview of samples.**

753 Specimen numbers and species identities are given as in the 16S phylogeny (Figure 2).  
754 “Genbank” indicates to the Genbank submission numbers of each specimen and marker (16S  
755 or 28S). Locality codes and names refer to sample localities during the four expeditions on  
756 Lake Baikal, except for the locality in the UK. S=south. Lat= latitude, Long=longitude.

757

758 **Table 2: Results of species delimitations with the PTP and the 4  $\theta$  method from the**  
759 **congruent molecular dataset.**

760 Results of the PTP algorithm are given in italics for each phylogenetic clade in the diagonal  
761 line, the results of the 4  $\theta$  rule for comparisons between phylogenetic clades are shown below  
762 the diagonal. Phylogenetic clades printed in bold fulfil the criteria of both methods. *t=*  
763 *tuberculata*.

764 For the PTP method, the statistical support for each particular phylogenetic group of  
765 individuals is provided. For the 4  $\theta$  rule, only the ratio for the genetic distance between  
766 phylogenetic clades is shown here; other calculations on which these ratios are based, are  
767 detailed in Table S1 (supplementary material).

768 \* = Singletons, to which the 4  $\theta$  method cannot be applied. \$ = Additional evolutionary  
769 genetic species detected with the PTP method. £ = Additional evolutionary genetic species  
770 detected with the 4  $\theta$  rule.

771

772 **Table 3: Ecological and geographical distribution data of genetic species from the**  
773 **congruent dataset.**

774 Genetic species and their pairs were defined from well-supported clades in the combined  
775 phylogenetic trees (using congruent sequence data from both 16S and 28S; Figure 2).  
776 Distribution data differentiating sister pairs are printed in bold. na= no data available;  
777 N=North, E=Eastern, C= Central, S=Southern, W= Western. Depth is water depth in meters.  
778

## 779 **Figures**

### 780 **Figure 1: Approximate position of sampling stations in Lake Baikal.**

781 Labels refer to the sampling stations in Table 1.

782

### 783 **Figure 2: Congruent phylogeny based on 16S and 28S.**

784 This phylogeny has been constructed with Maximum Likelihood and Bayesian methods on  
785 DNA sequences of 922 nucleotides each. Statistical support is shown above (PHYML,  
786 bootstrap values) and below (MrBayes, posterior probabilities) branches, respectively. *C. sp.*  
787 1 to *C. sp.* 4 are new species still awaiting formal description. Roman numbers refer to  
788 genetic species according to Table 2. Species printed in bold also show morphological  
789 differences. The coloured columns next to the tree show the type of sediment (A), depth (B),  
790 basin (C) and shore (D). Missing data are indicated in black.

791

### 792 **Figure 3: Results of the Principal Coordinates Analysis of genetic species defined by** 793 **the congruent molecular dataset and their ecological and geographic distributions.**

794 Identities of genetic species are similar to Figure 2. Species pairs from Table 3 are indicated  
795 by similar colours. Unpaired species are shown in black. If dots are labelled with several  
796 species names, these species share the same space in the coordination system. For all species  
797 shown, ecological data on sediment type and water depth were taken from Mazepova (1998;

798 see Table S2 for details), and geographical distribution data according to Table 3. Note that  
799 the new species *C. sp. 4* from Table 3 was not included in the analyses because no data on  
800 sediment type and water depth were available for this species from Mazepova (1998). *t =*  
801 *tuberculata*.

802

803 **Supplementary material:**

804 **Table S1: Results of evolutionary species delimitations with the 4  $\theta$  rule** (

805 Specimen numbers and species names refer to the 16S phylogeny (Figure 2).  $n^1$  = number of  
806 specimens for sister clade 1,  $n^2$  number of specimens for sister clade 2.  $\theta$  corresponds to the  
807 population mutation rate, having been corrected for the number of specimens.  $D$ = genetic  
808 distance.  $na$ = not applicable - calculations could not be conducted because only one sequence  
809 (singleton) was present per phylogenetic sister clade. Ratios of  $D/\theta$  larger than 4 fulfil the  
810 criteria of the 4 theta rule and are indicated in bold.

811

812 **Table S2: Comparison of ecological distribution data between our dataset and the data**  
813 **from Mazepova (1998).**

814 For the newly described species *C. sp. 1* to *C. sp. 4*, no data were available from Mazepova  
815 (1998).

816

817 **Table S3: Results of species delimitations with the PTP and the 4  $\theta$  method from 16S.**

818 Results of the PTP algorithm are given in italics for each phylogenetic clade in the diagonal  
819 line, the results of the 4  $\theta$  rule for comparisons between phylogenetic clades are shown below  
820 the diagonal. Phylogenetic clades printed in bold fulfil the criteria of both methods.  $t =$   
821 *tuberculata*. For the PTP method, the statistical support for each particular phylogenetic  
822 group of individuals is provided. For the 4  $\theta$  rule, only the ratio for the genetic distance

823 between phylogenetic clades is shown here; other calculations on which these ratios are  
824 based, are available from IS on request. \* = Singletons, to which the 4  $\theta$  method cannot be  
825 applied. \$ = Additional evolutionary genetic species detected with the PTP method. £ =  
826 Additional evolutionary genetic species detected with the 4  $\theta$  rule.

827

828 **Figure S1: 16S phylogeny.**

829 Statistical support is shown above (PHYML, bootstrap values) and below (MrBayes,  
830 posterior probabilities) branches, respectively. *C. sp. 1* to *C. sp. 4* are new species still  
831 awaiting formal description. Roman numbers refer to genetic species according to Table 2.  
832 Species printed in bold also show morphological differences. Specimens being also present in  
833 the combined 16S/28S phylogeny are indicated with a plus. The coloured columns next to the  
834 tree show the type of sediment (A), depth (B), basin (C) and shore (D). Missing data are  
835 indicated in black.

836

837 **Figure S2: 28S phylogeny.**

838 Statistical support is shown above branches as bootstrap values of 100%. Bayesian methods  
839 did not support the phylogeny whatsoever. Species names and Roman numbers refer to the  
840 species as delimited by 16S (Figure S2). For underlined specimens, no 16S data are  
841 available.

842

843 **Figure S3: Illustrations of valve morphology of the five genetic species within the classic**  
844 **morpho-species *Cytherissa tuberculata tuberculata*.**

845 A,B : *C. tuberculata tuberculata* I (loc. BK15). C,D : *C. tuberculata tuberculata* II (loc.  
846 BK8). E,F: *C. tuberculata tuberculata* III (loc. BT11). G,H : *C. tuberculata tuberculata* IV  
847 (loc. BK21). I,J : *C. tuberculata tuberculata* V (loc. BT18).

848 A, C, E, G, I: Right Valves, external views. B, D, F, H, J: Right Valves, internal views.  
849 Scales: 1 mm for E, G, I. 500  $\mu$ m for A, B, C, D, F, H, J. Please note that genetic species *C.*  
850 *tuberculata tuberculata* I can only be recognized by 16S (Table 1 & S3; Figure S1 & S4).

851

852 **Figure S4: 16S minimum spanning network.**

853 For each haplotype, the size of the circle is proportional to its frequency. Colours indicate the  
854 geographic origin of haplotypes from the three Baikalian basins and the UK, respectively.  
855 Species identities of haplotypes are indicated by coloured names and circles in the same  
856 colours around haplotypes. Species names refer to Figure S1.

857

858

859 **Figure S5: 28S minimum spanning network.**

860 For each haplotype, the size of the circle is proportional to its frequency. Colours indicate the  
861 geographic origin of haplotypes from the three Baikalian basins and the UK, respectively.  
862 Species identities of haplotypes are indicated by coloured names and circles in the same  
863 colours around haplotypes. Species names refer to Figure S2 or, if underlined, to the  
864 identification *sensu* Mazepova (1990) because no 16S data are available for these specimens.  
865 If several species names are shown next to a haplotype, then the latter is shared between these  
866 species. The most common haplotype is found in 20 different species.