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1 Exploring the Udoteaceae diversity (Bryopsidales, Chlorophyta) in the Caribbean region based on
2 molecular and morphological data

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10 Abstract:

11 The Udoteaceae family (Bryopsidales, Chlorophyta) is known to be highly diverse morphologically in
12 the Caribbean region, but only few studies have studied its genetic diversity. Using an integrative
13 taxonomic approach, this study aimed at (1) exploring the Udoteaceae species diversity using a
14 combination of five DNA-based species delimitation methods and morpho-anatomical data for
15 confirmation; (2) estimating the discriminatory power of traditional diagnostic characters using a
16 morphology-based clustering method and statistical analyses focused on the genus *Udotea*; and (3)
17 reconstructing the phylogeny of the family based on a multilocus analysis (*tufA*, *rbcL*, 18S rDNA). Our
18 results revealed strong congruence between species hypotheses across delimitation methods and
19 markers. Morpho-anatomical characters proved essential to validate these hypotheses, to assign
20 species names and to unveil new species. Morphological analyses led to relevant results for
21 accurately discriminating *Udotea* morphospecies. Siphon features and cortication were key
22 characters to define supra-specific groups and to revise the taxonomy of the genus *Udotea*.
23 Phylogenetic analyses confirmed the polyphyly of *Udotea*, *Rhipocephalus* and *Penicillus*, which led us
24 to propose a revised definition of *Udotea sensu stricto* based on both genetic and morphological
25 data. Finally, our study emphasizes the importance of combining genetic and morphological data for
26 the taxonomic revision of the Udoteaceae, but stresses the need of including more taxa from other
27 geographical regions to better resolve taxonomic issues.

28 Highlights:

- 29 • A species delimitation approach combining distance and tree-based methods is proposed
- 30 • High congruence is reached between methods and chloroplast markers
- 31 • A morphology-based clustering method proved relevant for discriminating the
32 morphospecies in the genus *Udotea*
- 33 • *Udotea*, *Penicillus* and *Rhipocephalus* revealed polyphyletic in our multilocus phylogeny
- 34 • The genus *Udotea sensu stricto* is redefined based on congruent genetic and morphological
35 data

36 Key words: Bryopsidales, Udoteaceae, *Udotea*, species delimitation, Multilocus phylogeny,
37 Morphological clustering

38

39 Declarations of interests: none

40 1. INTRODUCTION

41 Udoteaceae is a family of green macroalgae belonging to the order Bryopsidales (Chlorophyta). It
42 comprises both calcified and non-calcified species, and has a significant ecological function through
43 its contribution to primary production and carbonate fluxes. The Udoteaceae are particularly diverse
44 morphologically, the most among the bryopsidalean families. Their internal structure is siphonous
45 and characterized by a unique giant multinucleate cell that ranges from simple siphonous filaments
46 to complex multi-axial structures. They include tufts of uncalcified and free filaments (*e.g.*,
47 *Chlorodesmis*), brush-like calcified thalli (*e.g.*, *Penicillus*) or calcareous compact fan-shaped blades
48 (*e.g.*, *Udotea*). Although they occur in the Mediterranean Sea, Udoteacean species are mostly
49 tropical and particularly abundant and diverse in the Caribbean zone. Several authors have
50 investigated the North Atlantic marine flora over the last decades (see Wynne (2017) for a review),
51 and several species of *Udotea* were morphologically described by Littler and Littler (1990) and
52 recorded by Collado-Vides et al. (2009) in their revision of the genus diversity from Cuba and Mexico.
53 According to these works, only four out of the fourteen currently accepted Udoteaceae genera are
54 present in the tropical western Atlantic, with one of them endemic to this region (*Rhipocephalus*). All
55 genera included, twenty-four species have been listed, of which seventeen are only found in the
56 tropical western Atlantic and ten are restricted to the Caribbean region. However, most of these
57 species are based on morphological descriptions and genetic data is still unavailable or fragmentary,
58 a common trend in the Udoteaceae. Indeed, since J. Agardh (1887) described the family, the
59 taxonomy of the Udoteaceae has been established predominantly on morphological and anatomical
60 traits (Ernst, 1904; Farghaly, 1980; Gepp and Gepp, 1911; Littler and Littler, 1990). The most
61 significant taxonomical work on Udoteaceae is that of Gepp and Gepp (1911) based on material
62 sampled during the Siboga expedition to the East Indian archipelago. Life cycles and sexual
63 reproduction patterns were also the subject of a few studies but have not been reviewed since the
64 work of Meinesz (1980) and Vroom et al. (1998), the latter performed cladistics analyses on
65 morphological, anatomical and reproductive characters. The first phylogenetic analysis has been
66 realized using nuclear-encoded ribosomal DNA (nrDNA) (Kooistra, 2002). This study emphasized the
67 polyphyly of several genera among the Udoteaceae (*Udotea*, *Penicillus*, *Chlorodesmis*), which was
68 later confirmed by Lam and Zechman (2006), Curtis et al. (2008) and Coppejans et al. (2011) based on
69 the chloroplast *rbcL* marker, and by Verbruggen et al. (2009a, 2009b) based on multilocus analyses.
70 All the results, together with the presence of cryptic diversity and/or morphological variability,
71 illustrate how challenging the study of Udoteaceae can be and emphasize the need for a taxonomic
72 revision.

73 Sequence-based species delimitation approaches are efficient tools for discriminating species and
74 could help to better describe the Udoteaceae diversity and to resolve taxonomic ambiguities. A
75 number of methods have been developed to detect discontinuities in DNA sequence variation
76 associated with species boundaries. They have been applied at various taxonomic levels for the
77 Phaeophyceae (Montecinos et al., 2017; Silberfeld et al., 2013; Vieira et al., 2014), the Rhodophyta
78 (*e.g.*, Jesus et al., 2016; Guillemin et al., 2016; Pardo et al., 2014) and the Chlorophyta (Leliaert et al.,
79 2009; Sauvage et al., 2016; Zou et al., 2016). In this study, we have chosen five exploratory species
80 delimitation methods: the Automatic Barcode Gap Discovery (ABGD, Puillandre et al., 2012), the
81 General Mixed Yule Coalescent (GMYC) (Pons et al., 2006) and its Bayesian implementation, bGMYC
82 (Reid and Carstens, 2012), the Poisson tree process model (PTP, Zhang et al., 2013), and the Multi-
83 rate PTP (mPTP, Kapli et al., 2017). The association of these methods is interesting for the different

84 assumptions they rely on. On the one hand, ABGD is a distance-based method which aims at
85 identifying a barcode gap that delimits the intraspecific from the interspecific distances in the
86 distribution of genetic distances. On the other hand, the (b)GMYC and (m)PTP methods use
87 information from a phylogenetic tree and focus on branching and mutation rates to identify a shift
88 between speciation (between species) and coalescence events (within species). While (b)GMYC rely
89 on branching rates through time and thus require an ultrametric time-calibrated tree, (m)PTP relies
90 on the number of substitutions along branches, and, in the case of mPTP, accounts for divergent
91 intraspecific variation. The second group of methods (tree-based methods) assumes the monophyly
92 of the delimited species, which is not the case of the distance-based methods such as ABGD. To
93 converge towards robust species hypotheses, several authors strongly recommend using multilocus
94 datasets and assessing the congruence between several methods (Carstens et al., 2013; Carstens and
95 Knowles, 2007; Dupuis et al., 2012; Leliaert et al., 2014; Puillandre et al., 2012b; Rannala, 2015). The
96 analysis of non-genetic data is also recommended by many authors to discuss DNA-based species
97 hypotheses (Carstens et al., 2013; Carstens and Knowles, 2007; Fujita et al., 2012; Talavera et al.,
98 2013; Wiens, 2007).

99 Following these recommendations, we based our study on molecular data, using several genetic
100 markers to propose Primary Species Hypotheses (PSHs) and infer their phylogenetic relationship,
101 combined with morpho-anatomical data to propose Secondary Species Hypothesis (SSHs), following
102 an integrative taxonomy strategy. Our objectives were to: (i) explore the diversity of the Udoteaceae
103 in the Caribbean region, (ii) clarify species boundaries and (iii) produce a more accurate phylogeny of
104 the family. To reach these objectives, we (i) applied the five species delimitation methods described
105 above using two chloroplast markers; (ii) validated the resulting DNA-based species hypotheses using
106 cladistic analyses of morpho-anatomical traits, and (iii) reconstructed the phylogeny of the family
107 based on the analysis of the chloroplast *tufA* and *rbcl*, and the nuclear 18S rDNA markers.

108 2. MATERIAL AND METHODS

109 2.1 Sampling and study region

110 A total of 205 specimens of Udoteaceae from the Caribbean region were included in the study. Most
111 of the samples (127) were collected in the Lesser Antilles from 10 islands, including 102 specimens
112 from the scientific campaign PACOTILLES in 2015 (DOI, 10.17600/15005200) onboard the vessel
113 *Antea*, and 25 specimens collected in 2014 in Guadeloupe (Supplementary Figure A.1). All our
114 samples were collected by SCUBA from a total of 20 sites between the surface and 40m deep. Sites
115 were georeferenced and habitats described using bathymetric and substrata descriptors. When
116 possible, specimens were photographed on site prior to collection in order to document their *in-situ*
117 habits. Samples were sorted, labeled and photographed while fresh. Subsamples were preserved in
118 both 95 % ethanol and silica gel for later DNA analyses. Specimens were then pressed-dried as
119 herbarium vouchers and are currently housed at NOU in New Caledonia. The other 78 specimens
120 were collected and sequenced by collaborators of the National Museum of Natural History and the
121 School of Biosciences of the University of Melbourne, which enabled us to extend our geographical
122 coverage to most subregions of the Caribbean zone. The latter specimens are housed at PC in Paris
123 and GENT respectively (herbarium abbreviations follow Thiers (2016), continuously updated).

124 2.2 DNA extraction, amplification and sequencing

125 From the 127 Udoteaceae specimens collected in the Lesser Antilles, 114 samples were successfully
126 extracted using the DNeasy Plant Mini Kit (Qiagen Inc, Valencia, CA, USA) following a modified and
127 optimized protocol based on the manufacturer's instructions (available on request). Sequences for
128 two chloroplast and one nuclear genes were produced: *tufA* (approximately 800 base pairs -bp), *rbcl*
129 (approx. 1400 bp) and 18S rDNA (approx. 1300 bp) using previously published primers (Händeler et
130 al., 2010; Kooistra, 2002; Lam and Zechman, 2006; Verbruggen et al., 2009a) (Supplementary
131 material, Table A.1). The *rbcl* and 18S rDNA markers were sequenced in two fragments. PCR
132 reactions were conducted in a final volume of 25 μ L including of 12.5 μ L of AmpliTaq Gold 360
133 Master Mix (Applied Biosystems), 0.75 μ L of dimethylsulfoxide (DMSO), 1 μ L of bovine serum
134 albumin (BSA), 1 μ L of each primer (10 μ M), 2.5 μ L of DNA, and 6.25 μ L of ultra-pure water (PCR
135 programs available on request). The Sanger sequencing reaction was carried out using 20 μ L of PCR
136 product by Genoscreen (Lille, FRANCE). Sequences were edited in Geneious version 7.1.9
137 (<http://www.geneious.com>, Kearse et al., 2012) and aligned for each marker separately using the
138 MUSCLE algorithm available in the software. In addition to the sequences produced during this study,
139 Udoteaceae sequences obtained from collaborators (National Museum of Natural History: 42 *tufA*
140 sequences, and the School of Biosciences, University of Melbourne: 33 *tufA* and 6 *rbcl* sequences)
141 and GenBank (16 *rbcl* and 15 18S rDNA sequences) were added to the datasets. The detailed list of
142 specimens used and the accession numbers are recorded in Supplementary material (Table A.2).

143 2.3 Trees inference for species delimitation

144 The species delimitation methods were used for the analysis of the two chloroplast markers, *tufA*
145 and *rbcl*, known for their discriminatory power at the species level in green macroalgae (Leliaert et
146 al., 2014; Saunders and Kucera, 2010; Verbruggen et al., 2009a). The 18s rDNA was not used for
147 species delimitation due to the low intra and interspecific variability observed in preliminary analyses
148 (results available upon request). Both Bayesian ultrametric and Maximum Likelihood (ML) trees were
149 constructed to produce the two types of input required for tree-based delimitation methods.
150 Identical sequences (numerous for the *tufA* dataset) were removed using the Collapsetypes v4.6 perl
151 script (Chesters, 2013) to keep only one sequence for each haplotype, in order to prevent potential
152 analysis bias (e.g., calculation of an infinite coalescent λ and over-partition of the dataset; see Pons et
153 al. (2006) and Reid and Carstens (2012) and to decrease computation time (Kapli et al., 2017;
154 Talavera et al., 2013). *Codium taylorii* P.C. Silva (Codiaceae, Bryopsidales), *Caulerpa sertularioides*
155 (S.G. Gmelin) M. Howe (Caulerpaceae, Bryopsidales) and *Avrainvillea lacerata* J Agardh
156 (Dichotomosiphonaceae, Bryopsidales) were used as outgroup taxa for tree reconstruction but
157 removed before running the species delimitation analyses. Best substitution models were identified
158 under the Akaike information Criterion (AIC) implemented in jModelTest 2 (Darriba et al., 2012;
159 Guindon and Gascuel, 2003) as follows: GTR+I+G for *tufA* and HKY+I+G for *rbcl*.

160 Ultrametric trees were obtained using BEAST v. 1.8.3 (Drummond et al., 2012) through the CIPRES
161 web portal (Miller et al., 2010) from two independent runs of 50 and 120 million generations,
162 starting from a random tree and sampled every 5,000 and 12,000 generations for *tufA* and *rbcl*,
163 respectively. Because the null clock hypothesis was rejected for both datasets (Likelihood ratio test in
164 MEGA 6 (Tamura et al., 2013)), the BEAST analyses were run under a relaxed log-normal molecular
165 clock (Drummond et al., 2006) with a coalescent constant size tree prior as recommended by
166 Monaghan et al. (2009). The output of each run was checked in Tracer 1.5 (Rambaut and Drummond,
167 2007) to confirm the convergence of the Markov Chains Monte Carlo (MCMC), with effective sample

168 sizes all above 200. Outputs were then combined using Log Combiner (included in the Beast
169 package), removing the first 10 % generations as burn-in. The Maximum Clade Credibility Tree
170 (MCCT) was calculated using Tree Annotator (included in the Beast package).

171 The maximum likelihood analyses were run using RAxML (Stamatakis, 2014) through the CIPRES web
172 portal (Miller et al., 2010), using the rapid bootstrapping and search for best-scoring ML tree
173 algorithm. A multi-parametric bootstrap resampling with 1,000 iterations provided bootstrap
174 supports (Stamatakis, 2014).

175 The Bayesian MCCTs were used as the input tree for the GMYC method. The bGMYC (multitree)
176 analysis was run using a subsample of 100 trees from the Beast analyses for each marker. The PTP
177 method was applied to ML rooted trees and mPTP was applied on both ML trees and MCCTs.

178 2.4 Exploratory species delimitation and validation strategy

179 ABGD was performed through the website
180 (<http://www.wabi.snv.jussieu.fr/public/abgd/abgdweb.html>) and applied to both *tufA* and *rbcl*
181 alignments. The *rbcl* marker was analyzed as two distinct datasets (*rbcl5'* and *rbcl3'*) following the
182 two-fragment scheme of sequencing, in order to avoid potential bias in the calculation of genetic
183 distance due to different dataset sizes and/or lack of species representatives. Default values for all
184 the parameters were used, except for the X value, for which the default value returned partitions
185 with only one species. As advised on the ABGD website, we thus decreased the X value until we
186 obtained partitions with more than one species: the X-value was set to 1 for *rbcl5'* and *rbcl3'*, and
187 0.8 for *tufA*.

188 The GMYC method was applied using the "splits" package under the R environment (R Development
189 Core Team, 2017). For the likelihood ratio test, a *P-value* < 0.112 was seen as significant following the
190 revised model of Fujisawa and Barraclough (2013). The bGMYC method was also conducted in the R
191 environment using the "bGMYC" package (Reid and Carstens, 2012). After exploratory tests, the
192 method was run for 30,000 generations with the 15,000 first generations removed as burn-in for
193 *tufA*, and 60,000 generations with the 20,000 first generations removed as burn-in for *rbcl*. Sampling
194 was set every 100 generations for both analyses.

195 The PTP method was carried out on the Exelixis Lab web server (<http://sco.h-its.org/exelixis/web/software/PTP/index.html>). It was run for 500,000 generations with a thinning
196 value of 100 and a burn-in of 10%. MCMC convergence was visually checked before the
197 interpretation of the results as recommended by Zhang et al. (2013). The mPTP analysis was run
198 through the mPTP web server (<http://mPTP.h-its.org>) with the default settings.

200 The assessment of the species hypotheses provided by ABGD, GMYC and PTP consisted in a search
201 for common PSHs among the different methods. In case of conflict, the most prevalent partition (or
202 PSHs) found among the methods was preferred (*i.e.*, the partitions had to be shared at least by three
203 out of the five methods) in order to define a SSHs. We then used morpho-anatomical traits to
204 confirm the SSHs and assign species names.

205 2.5 Morpho-anatomical analyses

206 The morphological and anatomical observations were made on fragments of herbarium specimens
207 decalcified in HCL solution (5%) and observed under a microscope (Olympus BH-2) equipped with an
208 Olympus Camedia C-5050 digital camera (Tokyo, Japan). In order to validate the SSHs resulting from
209 the species delimitation analysis, and to assign them species names, up to five specimens per SSHs
210 were studied (depending on availability). Species identification followed the morphological keys of
211 Gepp and Gepp (1911) and Littler and Littler (1990). The morpho-anatomical characters assessed in
212 this study are summarized in Table 2.

213 A morphology-based delimitation analysis was also performed. The aim was to evaluate the
214 efficiency and robustness of the morpho-anatomical characters in discriminating morphospecies
215 compared to DNA-based species delimitation methods. Here, we consider morphospecies as entities
216 that can be identified based on a combination of both morphological and anatomical diagnostic
217 criteria. The analysis was focused on *Udotea* species, because the genus is predominant in the
218 Caribbean. The analysis was based on 57 individuals for which 22 morpho-anatomical variables (two
219 quantitative and 20 qualitative) were compiled in an encoded matrix (see Supplementary data A.1).
220 Information for type species and species not sampled in this study were extracted from Littler and
221 Littler (1990) and added to the matrix. Based on this dataset, we performed a cluster analysis using
222 the packages “cluster” and “ade4” in R. After exploratory tests, we applied a hierarchical clustering
223 (hclust) analysis with the “average” cluster method and an Euclidean calculation of distances to
224 discriminate the specimens. With the aim to obtain delimited clades of morphospecies, we applied a
225 95 % confidence clustering with an a priori number of species. Simultaneously, using the “pvclust”
226 package in R (Suzuki and Shimodaira, 2006) and under the same parameters (dendrogram topologies
227 were congruent between both methods), we estimated approximately unbiased p-values of the
228 nodes as computed by multiscale bootstraps resampling with 1,000 bootstrap replications. To further
229 observe the discriminating power of the diagnostic morpho- anatomical characters, a Hill-Smith
230 analysis was carried out. This multivariate analysis performs with continuous and multi-state discrete
231 characters (Hill and Smith, 1976). All the variables were thus integrated in a single analysis, whether
232 they are qualitative or quantitative.

233 2.6 Phylogenetic analysis of DNA sequences

234 Phylogenetic trees were produced using maximum likelihood and Bayesian inference implemented
235 on individual alignments as well as a concatenated multilocus matrix including *tufA*, *rbcl* and 18S
236 rDNA sequences. The resulting concatenated dataset included only one representative per SSHs.
237 Outgroup taxa were the same as those used in species delimitation analyses (Supplementary
238 material, Table A.2). The best partition scheme for the dataset was identified using *Partition Finder*
239 v1.1.0 under the Akaike Information Criterion (AIC). Results suggested a partition by genes and codon
240 positions for the chloroplast protein-coding genes *tufA* (GTR+I, GTR+I, GTR+G) and *rbcl* (5' fragment:
241 GTR+G, GTR+I, GTR+I+G; 3' fragment: JC, GTR+I, GTR+G), and a unique partition for the 18S rDNA
242 (GTR+I+G). The Bayesian phylogenetic analysis was performed in MrBayes v.3.2 (Ronquist and
243 Huelsenbeck, 2003) through the CIPRES portal. Two independent runs of four incrementally heated
244 chains were run for 50 million generations. The convergence of the Markov chain Monte Carlo
245 (MCMC) and the ESS values were checked in TRACER v.1.5 (Rambaut and Drummond, 2007). The
246 10 % first generations were discarded as “burn-in” prior to computing a consensus topology and
247 posterior probabilities. The ML analysis was conducted in RAXML (Stamatakis, 2014) on the
248 partitioned dataset under a GTR + GAMMA +I nucleotide model. Parameters included a rapid

249 bootstrapping and search for best-scoring ML tree algorithm, as well as 1,000 bootstrap replicates
250 (Stamatakis et al., 2008).

251 3. RESULTS

252 3.1 Sequence variability, species delimitation analysis, definition of the SSHs and assignment

253 A total of 162 *tufA* sequences (834 bp, 46 unique haplotypes, 272 variable sites), 91 *rbcl* sequences
254 (1322 bp, 40 unique haplotypes, 258 variable sites) and 62 rDNA 18S sequences (1248 bp, 47 unique
255 haplotypes, 197 variables sites) (from targeted specimens for the latter) were obtained. The *rbcl*
256 sequences were sequenced in two parts, the *rbcl* 5' part was 718 bp long with 155 variable sites, and
257 the *rbcl* 3' part was 604 bp long with 103 variable sites.

258 *tufA* dataset - Results obtained with the five species delimitation methods for the *tufA* dataset are
259 summarized in Fig. 1. With the single threshold model, the GMYC analysis resulted in 16 PSHs
260 (confidence interval of 12-19; Likelihood ratio-test (LRT) = 0.033), the bGMYC and ABGD analyses
261 suggested 15 PSHs, while PTP and mPTP delimited 20 (and 12 PSHs, respectively. The bootstraps
262 values supporting the delimited PSHs with the PTP are given in Supplementary material (Table A.3).
263 Focusing on differences in the partition results, GMYC separated LL057 from the rest of the clade 4,
264 while PTP split the same clade in four PSHs. PTP also split in two PSHs the clades 1, 11 and 12, and
265 grouped the clades 2 and 3 in a single PSH. The mPTP method grouped the clades 6, 7, 8 and 9 in a
266 single PSH.

267 *rbcl* dataset - Results obtained with the five species delimitation methods for the *rbcl* dataset are
268 summarized in Supplementary material (Figure A.2). The GMYC and ABGD methods both resulted in
269 15 PSHs (confidence interval of 3-42, LRT= 0.086, threshold time= - 0.0070 for GMYC). The bGMYC
270 and mPTP methods both produced 16 PSHs, whereas PTP produced 23 PSHs) (see Supplementary
271 Table A.4 for the PSHs *bs* values for PTP and bGMYC methods). The clade 7 was split in two PSHs by
272 bGMYC and PTP. The clade 10 was recovered as a single PSHs for all methods except PTP for which
273 the clade was split into five PSHs. In addition, PTP separated NOU087132 from the rest of the clade 6
274 and split the clade 3 in three PSHs. mPTP singled out HV405 from the rest of clade 9, DQ469318 from
275 the rest of clade 6, and HV02674 from the rest of clade 13. mPTP also grouped in a single PSHs the
276 clades 2+3 and 16+17.

277 SSHs definition - Overall, our analyses produced relatively congruent PSHs between the different
278 delimitation methods and both marker datasets (Table 2). However, the GMYC multiple-threshold
279 method was not considered because it provided much higher number of PSHs, compared to the
280 other methods. Besides, we only presented the results obtained using the MCCTs for the mPTP
281 method (but see Supplementary data A.2 and A.3 for the results of mPTP applied on ML rooted
282 trees). Despite several tests with ML rooted trees, the number of PSHs obtained were surprisingly
283 low (respectively 9 and 5 for *tufA* and *rbcl* dataset), we decided to not include these results in the
284 PSHs/SSHs discussion. ABGD, GMYC, bGMYC and mPTP provided similar partitions and are more
285 conservative than PTP, generally defined more PSHs for both datasets. Seven and five of the 15 PSHs
286 were recovered by all five methods for *rbcl* and *tufA*, respectively. All PSHs were supported by at
287 least three of the five delimitation methods, which led to the definition of 18 SSHs (*i.e.*, previously
288 identified as clades). Among these, 12 SSHs were represented in both datasets. Some SSHs could not

289 be compared between the two datasets as some sequences were missing for one or the other
290 marker.

291 Morpho-anatomical assignment of the SSHs – Based on morpho-anatomical observations, 15 out of
292 the 18 SSHs were assigned a known species name (Figures 1 and A.2). The three remaining SSHs
293 could not be identified either because of insufficient material for morpho-anatomical observations
294 (SSHs 1 and 4) or because the observed characters did not match any known species (SSH 5).

295 3.2 Independent morpho-anatomical analysis of the *Udotea* morphospecies

296 This part of the study was focused on morphology-based delimitation of species in the genus *Udotea*,
297 investigated through two different analyses: morphological-based clustering and multivariate
298 analysis. The discrimination of the specimens and the morphospecies clusters obtained under an *a*
299 *priori* number of species (18 or 22 depending on the consideration of species forms and varieties or
300 not) are illustrated on Figure 2. Most of the specimens were grouped in mostly well-supported
301 clusters containing one of the reference species described by Littler and Littler (1990); those that did
302 not, appeared to be juvenile forms (*e.g.*, NOU087081, NOU087164). Noticeable incongruences
303 included *Udotea cyathiformis* var. *flabellifolia* which clustered separately from *Udotea cyathiformis*
304 var. *cyathiformis* and its forms, and instead grouped with *Udotea conglutinata*. *Udotea cyathiformis*
305 var. *flabellifolia* is indeed markedly different from other forms and varieties of the species (fan-
306 shaped blade for the variety “*flabellifolia*” versus the cup-shaped blade of the variety “*cyathiformis*”).
307 The two species *Udotea luna* and *U. looensis* also grouped together in a single cluster, as well as
308 *Udotea spinulosa* and *U. spinulosa* f. *palmettoidea*.

309 Beyond morphospecies level, the multivariate analysis considered all morpho-anatomical variables
310 (Supplementary Figure A.3) and clearly recovered specimens in three groups (component axes 1 and
311 2 explained 58.23% of total variability). This subdivision in three groups was also very clear in the
312 independent analysis of two variables: the cortication and siphon features (Figure 3a, b). In the
313 cortication analysis, all the specimens were perfectly grouped according to the three states that were
314 considered: absence of cortex (1), complete cortex (2) and incomplete cortex (3). For siphon
315 features, the five states that were considered clustered in three groups as follow: the first group
316 matched the “absence of cortex” group and included species with smooth siphons (state 1) or with a
317 few basal scattered protuberances only on siphons from the blade/stipe basal transition zone (state
318 2); the second group matched the “complete cortex” group and included species with appendages
319 (state 5); the third group matched the “incomplete cortex” group and included species with a few
320 scattered protuberances on blade siphons, most of the time only on exposed areas (state 3) and
321 species with numerous protuberances all along blade siphons (state 4).

322 The groups highlighted in the multivariate analysis were also distinct on the dendrogram resulting
323 from the morphology-based clustering analysis (Figure 2 and Table 3). Non-corticated species (C)
324 were distinctively separated from completely corticated species (B) and incompletely corticated
325 species (A). Only one species did not follow this classification and was recovered in the corticated
326 group on the dendrogram reconstruction but not in the multivariate analysis (*U. wilsonii*, with
327 protuberances).

328 3.3 Species phylogeny

329 Phylogenetic analyses of individual markers produced highly congruent topologies for chloroplast
330 genes. The topology of the 18S rDNA gene tree showed polyphyly at the species level, although not
331 supported. However, the three gene trees all confirmed the polyphyly of the udoteacean genera
332 (individual gene trees are available on request to the first author). ML and Bayesian analyses, applied
333 to concatenated alignment (*tufA*, *rbcl* and 18S rDNA), produced similar topologies, except for one
334 node in clade D (Figure 4) that was not supported by the ML analysis (bs: 26) and absent in the BI
335 analysis. The BI tree was generally better supported than the ML tree. We considered nodes with bs
336 values > 95 and Posterior Probability (PP) > 0.98 as highly supported, and nodes with bs values
337 comprised between 95 and 60 and PP between 0.98 and 0.70 as moderately supported. Below these
338 values, nodes are considered not supported.

339 In both trees, the representative of Udoteaceae included in this study formed a fully supported clade
340 (clade A, Figure 4), but all genera represented by at least two species appeared polyphyletic. The
341 Udoteaceae was divided in two main subclades: clade B (moderately supported, bs: 79; PP: 0.70)
342 with four species of *Udotea* including the type species of the genus (*U. flabellum*), and clade C
343 (strongly supported, bs: 100, PP: 1) with the rest of the species. Remaining *Udotea* species appeared
344 in both clades D (strongly supported, bs: 99; PP: 1) and G (moderately supported bs: 65; PP: 0.85).
345 Three species of *Penicillus* clustered in Clade F (moderately to strongly supported, bs: 88; PP: 1) while
346 the fourth *Penicillus* species clustered in Clade D with *Udotea* and *Rhipocephalus* species.

347 4. DISCUSSION

348 4.1 Diversity of the Udoteaceae in the Caribbean region

349 The DNA-based species delimitation results highlighted 18 different SSHs in our Caribbean dataset.
350 We assigned species names to 15 SSHs using morpho-anatomical observations. These 15 taxa were
351 previously recorded by Gepp and Gepp (1911) and Littler and Littler (1990) and belong to *Udotea* (8),
352 *Penicillus* (4), *Rhipocephalus* (2), and *Rhipidosiphon* (1). One species may be new to science (clade 5
353 in Figures 21 and A.2) as its morpho-anatomical characters did not match any of the known species,
354 but we recommend the prior revision and redefinition of the genus *Udotea* before this species can be
355 described with confidence. Two other species (clades 1 and 4, Figure 1) could not be identified
356 because no specimen was available for morpho-anatomical analyses. Eight of the *Udotea* species
357 described by Gepp and Gepp (1911) and Littler and Littler (1990) were not found during our survey
358 and no sequence was available in public DNA databases. It cannot be excluded that some of these
359 species could correspond to clades 1 and 4. Additional effort is required to sample these species and
360 check their taxonomic status. Among all species, 16 were present in the Lesser Antilles, thus
361 highlighting the diversity of this sub-region.

362 4.2 Variability and relevance of the genetic markers used

363 The results of the species delimitation analyses strongly rely on the selection of the genetic markers.
364 In this study, we selected markers based on their known variability and their different evolutionary
365 rates (Leliaert et al., 2014; Saunders and Kucera, 2010; Verbruggen et al., 2009a). Our results
366 demonstrated that the *tufA* and *rbcl* chloroplast markers are well suited to study the Udoteaceae at
367 species level. The *tufA* sequences contained more variable sites than *rbcl* ones (32.61% versus
368 19.51 %). The first fragment of the *rbcl* gene (*rbcl* 5') is slightly more variable (21.59% of variable
369 sites) than the entire *rbcl* marker (19.51%) or the *rbcl*3' fragment (17.05%), contrary to what has

370 been found in other Bryopsidales families (Saunders and Kucera, 2010): this pattern needs to be
371 confirmed in other lineages of Udoteaceae. Chloroplast genes have been widely used for barcoding
372 purposes in the study of green macroalgae, *i.e.*, to assign unknown specimens to known species
373 (“specimen identification”, *sensu* Collins and Cruickshank, 2012). Here, we show that they are also
374 suitable markers to propose species hypotheses (“species delimitation”, *sensu* Collins et al., 2012). In
375 contrast, the nuclear 18S rDNA gene appeared less variable than the chloroplast markers and was
376 not a relevant choice for species delimitation methods. The insufficient variability of the 18S rDNA at
377 the species level has already been shown for another bryopsidalean family, the Caulerpaceae (Kazi et
378 al., 2013). Nevertheless, the nuclear 18S rDNA proved useful for phylogenetic analyses, as already
379 suggested by Kooistra et al. (2002) for *Halimeda* and Kazi et al. (2013) for *Caulerpa*, and applied for
380 multilocus analyses of Bryopsidales lineages (Verbruggen et al., 2009a, 2009b). We believe that our
381 results emphasize the need of markers from different genomic compartments to better understand
382 species diversity and their phylogenetic relationships. Indeed, combining markers from different
383 cellular compartments or from unlinked nuclear markers allows the identification of any lack of
384 genetic divergence or potential incomplete lineage sorting or introgression events that would
385 otherwise not be detected with a single locus (Carstens et al., 2013; Carstens and Knowles, 2007;
386 Leliaert et al., 2014; Rannala, 2015).

387 4.3 Efficiency and limits of the species delimitation methods

388 In our study, the GMYC single-threshold method produced results congruent with other methods - if
389 not similar- and with the morpho-anatomical observations. The high similarity between GMYC and
390 ABGD in our study is worth pointing out as these two methods are usually seen as complementary
391 for the contrasting results they produce (Montecinos et al., 2017; Puillandre et al., 2012b). However,
392 the multiple-threshold method produced partitions with much more species hypotheses compared
393 to other methods, which is a tendency emphasized in previous studies (Esselstyn et al., 2012;
394 Guillemin et al., 2016; Modica et al., 2014; Montecinos et al., 2017; Tang et al., 2014).

395 The results obtained with bGMYC did not differ from the other methods and were consistent
396 between both the *tufA* and the *rbcL* datasets. However, the computation of the bGMYC rates
397 unveiled more Yule events (speciation) than coalescent events, which could have had an impact on
398 the analysis (Reid and Carstens, 2012). In the present study, because our dataset included several
399 genera and numerous species with low intraspecific variation, it is possible that coalescent events
400 have been under-represented. The bGMYC results need to be interpreted carefully and in
401 conjunction with other methods.

402 The results of the distance-based method, ABGD, were not very different from the other methods
403 with partition schemes similar to at least one of the tree-based method for each marker. However,
404 the method produced contrasted genetic distance distributions for the two markers. There was a
405 clear barcode gap for *tufA*, which was not the case for *rbcL*. For *rbcL*, one of the distributions
406 displayed two clear gaps but the other didn't show any gap. There are several possible explanations
407 for the absence of a unique and clear barcode gap, which include limited species sampling or variable
408 rates of evolution among species or genera. Incomplete lineage sorting or introgression could also
409 explain an absence of barcode gap (Talavera et al., 2013).

410 For both markers, the PTP method tended to overestimate the number of species while the mPTP
411 method appeared more congruent with the other methods (similar PSHs) and morphology. The

412 differences between the two methods can be explained by the ability of mPTP to consider variability
413 in exponential distributions while PTP cannot accommodate intraspecific diversity. However, the
414 mPTP method provided partitions congruent with the other methods only when using MCCTs:
415 partitions obtained with ML trees largely underestimated the number of PSHs, compared to the
416 number of SSHs finally retained. Both PTP and mPTP methods were primarily designed for non-
417 ultrametric trees, so it remains unclear why PTP accommodated both ultrametric (MCCTs) and non-
418 ultrametric (ML) trees while the mPTP methods failed with non-ultrametric trees.

419 Combining different delimitation methods was thus necessary to balance their respective
420 assumptions, bias or limitations, and to search for congruence among theoretically different models.
421 In the present study, tree-based methods were relatively congruent (whether they rely on
422 ultrametric tree or not), and also concordant with the distance-based method, ABGD. This
423 congruence reinforces the reliability of the resulting SSHs (Fujita et al., 2012; Ross et al., 2010).

424 4.4 Leverages and limits of involving morphological data to assign species names

425 Morpho-anatomical observations were used to assess SSHs, to assign species names, and to
426 eventually determine if some of the genetically defined PSHs had never been described before and
427 may correspond to new species.

428 However, not all the PSHs have been assigned a species name. This can be explained by the absence,
429 fragmentary state or inaccuracy of the available information for the family (morphological or
430 genetic). Hence our study was hindered by (1) insufficient or unavailable voucher material for
431 morpho-anatomical observations; (2) the limited number of reference sequences, particularly lacking
432 for holotypes, to properly allow molecular identification; and (3) unreliable species identification for
433 available sequences, often due to insufficient morpho-anatomical analysis (relying exclusively on
434 gross morphology).

435 Difficulties are also encountered from the morpho-anatomical characters themselves. Indeed, the
436 morphological identification of Udoteaceae species can easily be misled by different factors such as
437 *phenotypic* plasticity, cryptic diversity, life stages, observation interpretability, etc. Besides, it has
438 been shown that a large set of specimens is required to cover the range of species polymorphism
439 (see Wiens and Servedio, 2000) and therefore to accurately describe species, which might not always
440 have been the case for the description of some Udoteaceae species. When morphological variability
441 is large, species identification is more difficult because only a few anatomical characters remain
442 relevant to discriminate and diagnose the species. For instance, we observed that the quantitative
443 variables (*e.g.*, size of filaments) tend to follow a continuum, meaning that size ranges overlap
444 between species rather than being clearly distinct (see also Collado-Vides et al., 2009). A
445 morphometry study, such as those realized on the genus *Halimeda* (Verbruggen et al., 2005a,
446 2005b), referencing the values of the continuous traits of Udoteaceae species could be relevant to
447 further analyze these characters beyond their overlapping measure ranges. Finally, some
448 morphological discrete traits can also be interpreted differently according to the observer (Fujita and
449 Leache, 2011) and the assessment of a form (*e.g.*, the shape of the blade appendages apices) can be
450 very subjective. That leads to even more confusion when species identification relies on a single
451 character or when this latter may vary among or within specimens.

452 In the present study, we had no case of erroneous species descriptions due to the presence of cryptic
453 diversity, or morphological variability, as the number of DNA-based species hypothesis was
454 congruent with morphological identification. However, we were not able to confirm all species of
455 Caribbean Udoteaceae listed in the literature because of the lack of genetic information for some
456 morphospecies or the absence of specimens for morpho-anatomical observations of some PSHs.

457 4.5 Relevance of morphological characters to discriminate *Udotea* species

458 The *Udotea* morpho-anatomical cluster analysis (Figure 2) produced well supported results which
459 largely matched with genetically delimited species hypotheses. This demonstrates the ability and
460 potential of the morphological characters for delineating *Udotea* species. The studies that used
461 morphological-based methods often found discordance with molecular results (Barrett and
462 Freudenstein, 2011; Doan and Castoe, 2003; Wiens and Penkrot, 2002). This situation may explain
463 why morphological traits analyses are under-used for delimiting species in recent molecular studies
464 (Wiens, 2007; Wiens and Penkrot, 2002). Here, we would like to emphasize the relevant contribution
465 of morphology to integrative taxonomy approaches, and to promote the value of reassessing
466 morphological diagnostic characters as already pointed out (Cianciola et al., 2010; Vieira et al., 2014).
467 We observed that the combination of several morpho-anatomical characters was relevant to
468 accurately delimit species. However, each character considered independently was relevant at higher
469 taxonomic level, such as groups of species or genus, but not at the species level. Finally, we
470 demonstrated that the cortication and the blade siphon features were relevant variables to explain
471 morphospecies discrimination. Taken alone, these two variables were sufficiently effective to explain
472 the clustering pattern observed for *Udotea* in both the multilocus and the multivariate analyses.

473 Nevertheless, from our results, we argue that a nomenclatural clarification of the term “appendages”
474 is needed to better understand the distinctiveness of *Udotea* species (as currently defined) and
475 acquire more indicators for the necessary redefinition of the genus. We propose to consider under
476 the term “appendages” only the well-developed secondary structures that are dichotomously divided
477 or lobed with numerous tips, and lead to a cortication (different from “protuberances”, which only
478 consist of outgrowths). Through the redefinition of these terms, it becomes easier to highlight the
479 discriminatory power of the siphon features and associate this latter to the cortication variable (see
480 Table 3 for the correlation). These both variables stand out from other morpho-anatomical
481 characters for discriminating *Udotea* sub-lineages and may be relevant indicators for future
482 taxonomic revision.

483 4.6 Phylogenetic relationship and taxonomic revision

484 The polyphyly of *Udotea* and *Penicillus*, that had been shown in previous molecular phylogenies
485 (Curtis et al., 2008; Kooistra, 2002; Lam and Zechman, 2006; Verbruggen et al., 2009a) was retrieved
486 in this study with also, for the first time, the polyphyly of the genus *Rhipocephalus*.

487 Traditional classification at the genus level relies on gross morphological observations which do not
488 agree with molecular phylogenies. For instance, the flabellate fan-shaped blade species have
489 traditionally been assigned to *Udotea* while DNA analyses showed that this form emerged in
490 genetically distinct clades and sometimes even grouped with other blade-morphologies (e.g., the
491 flabellate fan-shaped *Udotea conglutinata* closely related to the brush-like *Penicillus capitatus* in
492 clade D (Figure 4).

493 In our phylogenetic analyses, the Caribbean representatives of the Udoteaceae were split in two
494 main supported subclades, the first one being composed of *Udotea* species only, and the second
495 subclade regrouping sequences of different genera.

496 Furthermore, the split of the genus *Udotea* in several lineages was always supported, regardless of
497 the type of data used (molecular or morpho-anatomical), suggesting that it is more a taxonomical
498 problem rather than a genetic issue. Based on our results, we consider as *Udotea sensu stricto* (s. s.),
499 the group of species clustered in clade B of our concatenated phylogeny, which also includes the
500 type-species of the genus, *Udotea flabellum* (J. Ellis & Sollander) M. Howe. Species included in
501 *Udotea s.s.* appeared to be morphologically distinct and to have exclusive characters compared to
502 the other taxa traditionally assigned to *Udotea*. Species of *Udotea s.s.* all share similar habits with a
503 stocky thallus, a thick and strictly fan-shaped blade with a strong calcification, a well-developed
504 rhizoid system and siphons of moderate size ($\leq 80 \mu\text{m}$ for both stipe and blade filaments). The
505 strongest diagnostic character is the presence of appendages (dichotomously divided or lobbed with
506 rounded knobs) on the filaments of the blade which leads to cortication. This newly redefined
507 *Udotea s.s.* clade then corresponds to the corticated lineage mentioned by Kooistra (2002). In
508 addition to the four species included in clade B of our phylogeny (*Udotea occidentalis*, *U. dixonii*, *U.*
509 *dotyi* and *U. flabellum*), we propose to also include in *Udotea s. s.* the following species: *U. norrisii*,
510 and the Indo-Pacific *Udotea argentea* and *U. geppiorum*. From nuclear-based phylogeny and
511 morphological observations, Kooistra (2002) also considered *Udotea norrisii* among the ancestral
512 species (equal cortication of blade and stipe) along with *Flabellia petiolata*, *U. dotyi* and *U. flabellum*.
513 However, he excluded *U. argentea* from this group and rather considered its appendages as reduced
514 small stumps. Thus, our assumptions based on morphological agreement only need to be confirmed
515 with multilocus DNA analysis.

516 The remaining taxa included in our phylogeny all clustered in clade C. The morphology of these taxa
517 is variable, with filaments either assembled in single blade (fan- or cup-shaped), in several blades
518 attached to a main axis, or free in brush-like forms. These taxa have in common the absence of
519 appendages on the blade filaments. All the specimens clustered in clade C currently assigned to
520 *Udotea* are distinguishable from *Udotea s.s.* for their more delicate and thinner appearance, and
521 their wider filaments (largest siphons size in species with smooth filaments). All taxa of clade C need
522 to be taxonomically revised, particularly at the genus level. Kooistra (2002) suggested that all these
523 species could be considered as part of the same genus. Our phylogenetic results and morpho-
524 anatomical analyses rather suggest that species in clade C could be re-classified in several distinct
525 genera. For example, although clade D contains variable thallus morphologies, all the taxa have
526 smooth blade siphons. Similarly, taxa in clade F all have the same distinctive thallus morphology
527 (brush-like capitulum) and smooth blade filaments. Similarly, more genetic data is needed to confirm
528 if *Udotea* species with protuberances cluster with *U. spinulosa* (only species for which genetic data is
529 available). However, we believe that more species need to be studied before a taxonomic revision of
530 this group is proposed.

531 Our study highlights the need for in-depth taxonomic revision of the family, especially above the
532 species-level. Ideally, this revision should include all the taxonomical representatives of the family
533 across their distribution range, particularly the Indo-Pacific region for which little data is available.

534 5. CONCLUSION

535 Based on an integrative taxonomical approach, using genetic multilocus and morphological data
536 inferred from a taxa rich dataset, (i) we explored the diversity of the Udoteaceae from the Caribbean
537 region using five DNA-based species delimitation methods and morpho-anatomical analyses, (ii) we
538 produced a phylogeny of the family highlighting the polyphyly of most genera, and (iii) we proposed
539 a revised morphological description of the genus *Udotea* s. s. Our results demonstrate the adequacy
540 of the chloroplast markers (*tufA* and *rbcl*) for delimiting species. We emphasize the importance of (i)
541 searching for congruence between several species delimitation methods and markers, and (ii) using
542 independent data (here, morpho-anatomical) to increase the robustness of the species hypotheses,
543 and facilitate species name assignment. Performed independently, morpho-anatomical analyses led
544 to relevant results and emphasized the discriminatory power of morpho-anatomical characters to
545 delineate *Udotea* morphospecies. We demonstrate that morpho-anatomical characters represent
546 key data and helpful indicators in the process of taxonomical revision (at least at the genus level).
547 The polyphyly of the Udoteaceae genera was confirmed once more and clearly appeared as a
548 taxonomical issue rather than erroneous genetic signal. The redefinition of the genus *Udotea sensu*
549 *stricto* was achievable through the combination of both genetic and morphological information.
550 However, to resolve the Udoteaceae taxonomical issues in depth, a global study is necessary, and it
551 should ideally include all the taxonomical representatives of the family across their distribution
552 range, particularly the Indo-Pacific region.

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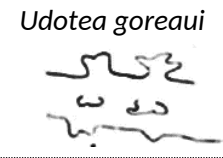
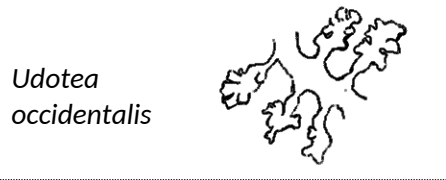
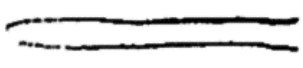
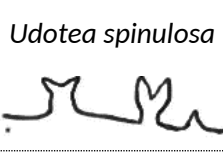
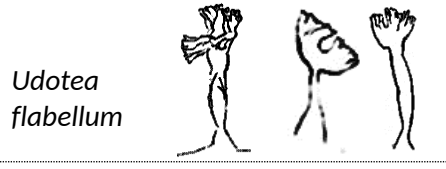
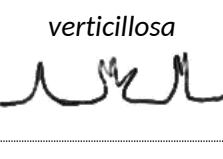
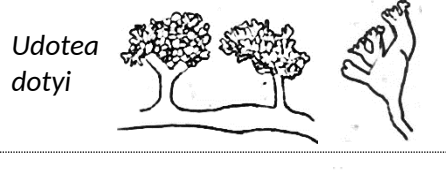
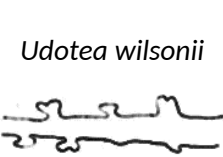


Table 1: Anatomical characters assessed for the identification of *Penicillus*, *Udotea* and *Rhipocephalus* species

Genera	Anatomical observations	
	Stipe filaments	Blade filaments
<i>Penicillus</i>	<ul style="list-style-type: none"> • Diameter • Appendage aspect (stalk, apices) 	<ul style="list-style-type: none"> • Diameter • Aspect (moniliform, tortuous or smooth) • Presence and narrowness of the constrictions above the dichotomies
<i>Rhipocephalus</i>	<ul style="list-style-type: none"> • Diameter • Appendage aspect (stalk, apices) 	<ul style="list-style-type: none"> • Diameter • Constant or degressive size of the filament from base to apex • Presence of dichotomies • Presence of constriction above dichotomies
<i>Udotea</i>	<ul style="list-style-type: none"> • Diameter • Appendage aspect (stalk, apices, dichotomous division) 	<ul style="list-style-type: none"> • Diameter • Aspect (moniliform, tortuous or smooth) • Presence of dichotomies • Presence of constriction above dichotomies • Presence of appendages and aspect (stalk, dichotomously division and apices)

Table2: Summary of the number of common PSHs between methods and markers. The PSHs obtained for *tufA* are indicated in red and the ones for *rbcl* in blue. Total number of PSHs for each methods and markers are referred in brackets.

<i>tufA</i> ; <i>rbcl</i>	GMYC (16 ; 15)	bGMYC (15 ; 16)	MLPTP (20 ; 23)	MPTP (12 ; 16)
ABGD (15 ; 15)	13 ; 15	15 ; 14	9 ; 8	11 ; 5
GMYC (16 ; 15)		13 ; 14	9 ; 8	7 ; 11
bGMYC (15 ; 16)			9 ; 11	11 ; 7
MLPTP (20 ; 23)				5 ; 5

Table 3: Overview of the three morphological main groups of *Udotea* species (A, B and C) defined through the morphological-based clustering and the multivariate analysis and also retrieved in the phylogeny (for the one for which molecular data were available). For each group, the morphological states of cortication and siphon features are detailed, and the species list, with illustrations of the corresponding siphon features.

Groups of <i>Udotea</i> species	A	B	C
Cortication state	Incomplete cortication (state 3)	Cortication (complete) (state 2)	No cortication (state 1)
Blade siphons features	Protuberances (state 3+4)	Appendages (state 5)	Smooth (state 1) [or a few protuberances at the transition zone blade-stipe] (state 2)
Corresponding species and siphons features illustrations	<i>Udotea goreau</i> 	<i>Udotea occidentalis</i> 	<i>Udotea cyathiformis</i> <i>Udotea conglutinata</i> <i>Udotea unistratea</i> <i>Udotea caribaea</i> <i>Udotea fibrosa</i> <i>Udotea abbottiorum</i> <i>Udotea luna</i> <i>Udotea looensis</i> <i>Udotea sp2</i> 
	<i>Udotea spinulosa</i> 	<i>Udotea flabellum</i> 	
	<i>Udotea verticillosa</i> 	<i>Udotea dotyi</i> 	
	<i>Udotea wilsonii</i> 	<i>Udotea dixonii</i> 	
		<i>Udotea norrisii</i> 	

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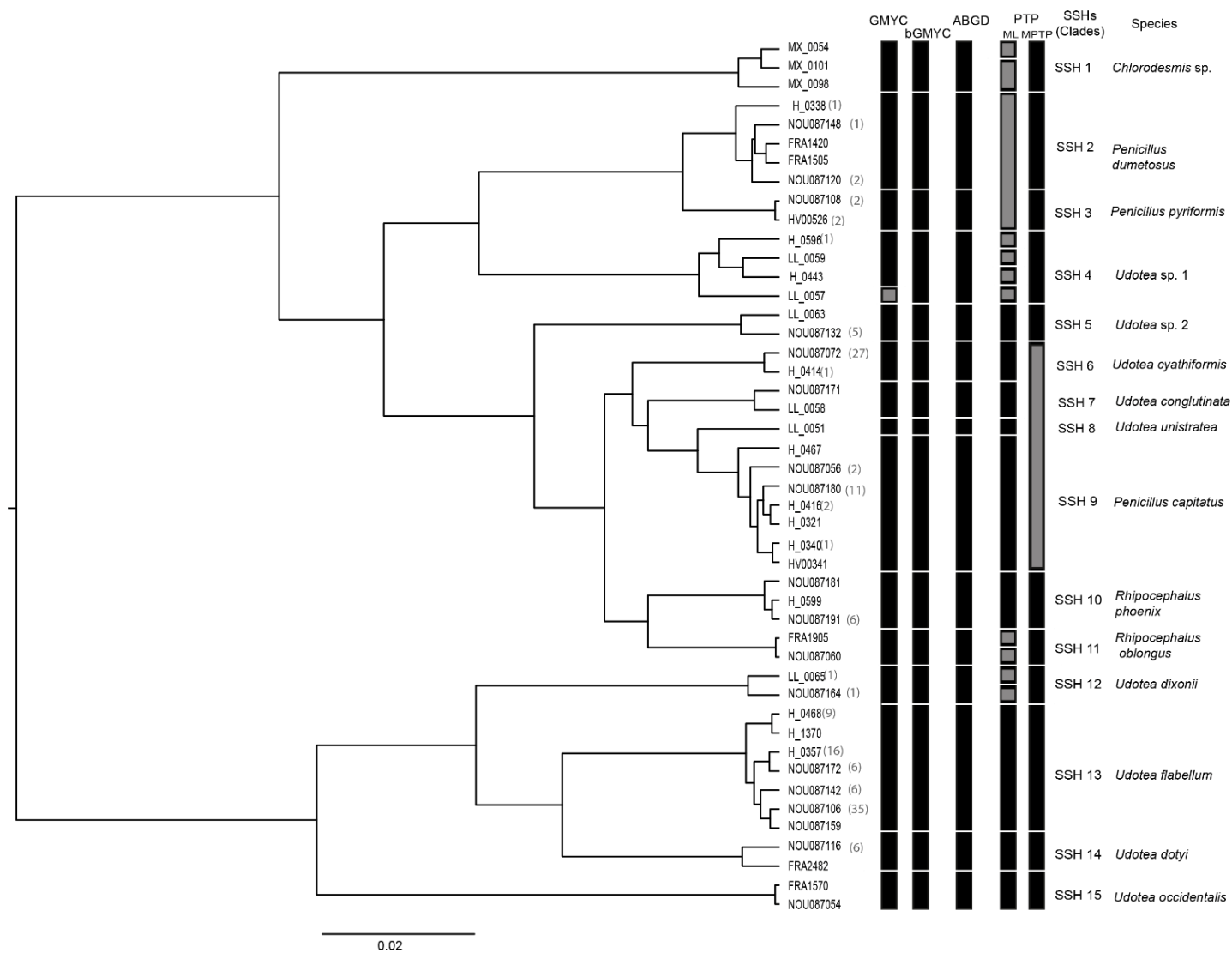


Figure 1: Species delimitation results obtained using the five methods (ABGD, GMYC, bGMYC, PTP and mPTP) applied to the *tufA* dataset. The tree is the maximum clade credibility tree from the BEAST analysis. Black bars indicate partitions retained as SSHs and indicated in right column as well as the species identification from morpho-anatomical observations. Grey bars indicate different partition schemes not retained. Numbers in brackets indicate the number of identical haplotypes in the initial dataset.

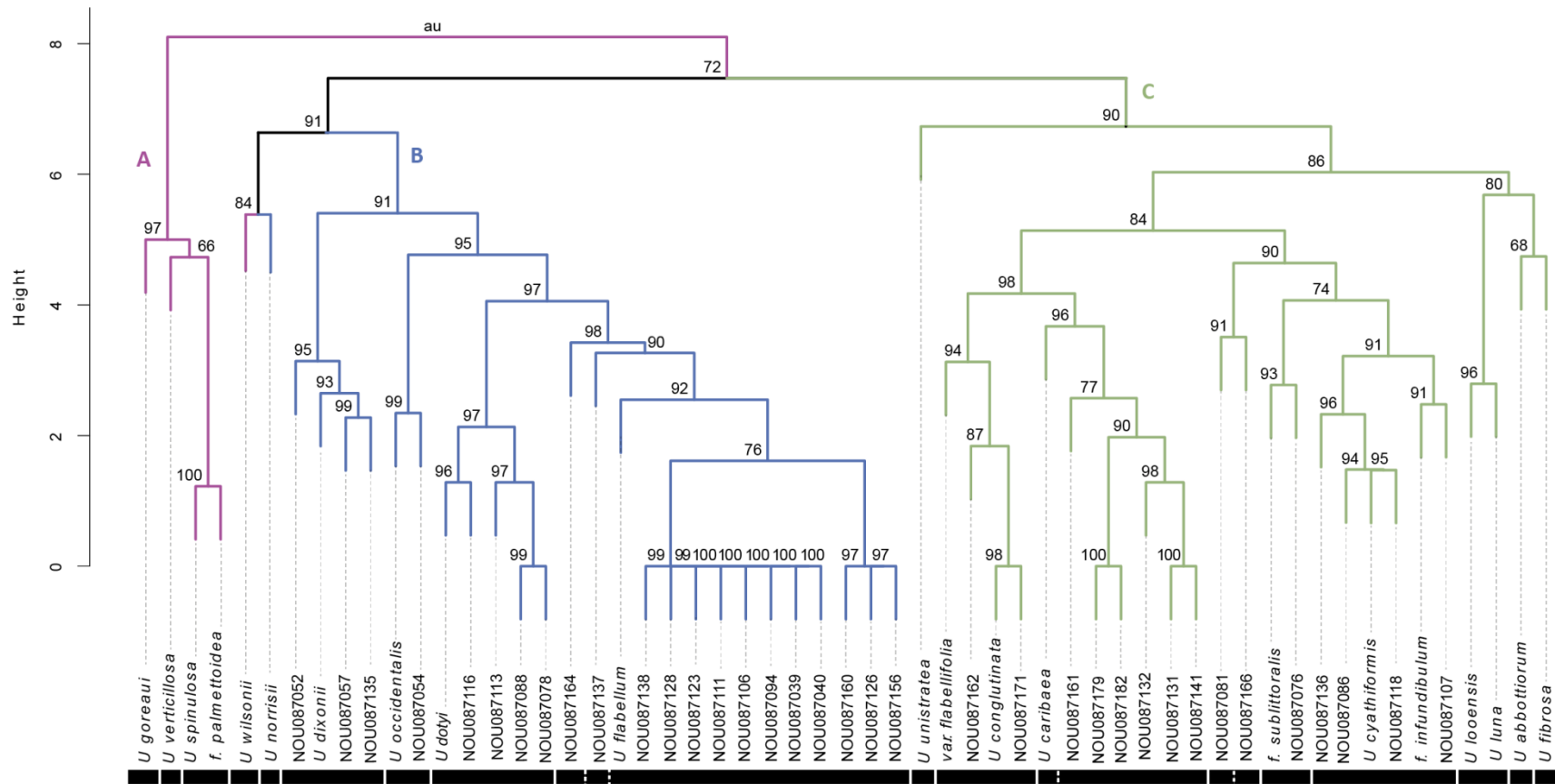


Figure 2: Dendrogram representation of the morpho-anatomical clustering (hclust analysis) applied on *Udoetea* specimens. The delimited morphospecies clusters are indicated in the timeline with a priori 18 (continuous lines) or 22 (dotted lines) number of species. Node values indicate approximately unbiased p-values. Different coloration of branches transcribes the three main groups of specimens (A, B, and C) highlighted from all the morpho-anatomical characters (detailed in Supplementary data A.1) and described in Table 3.

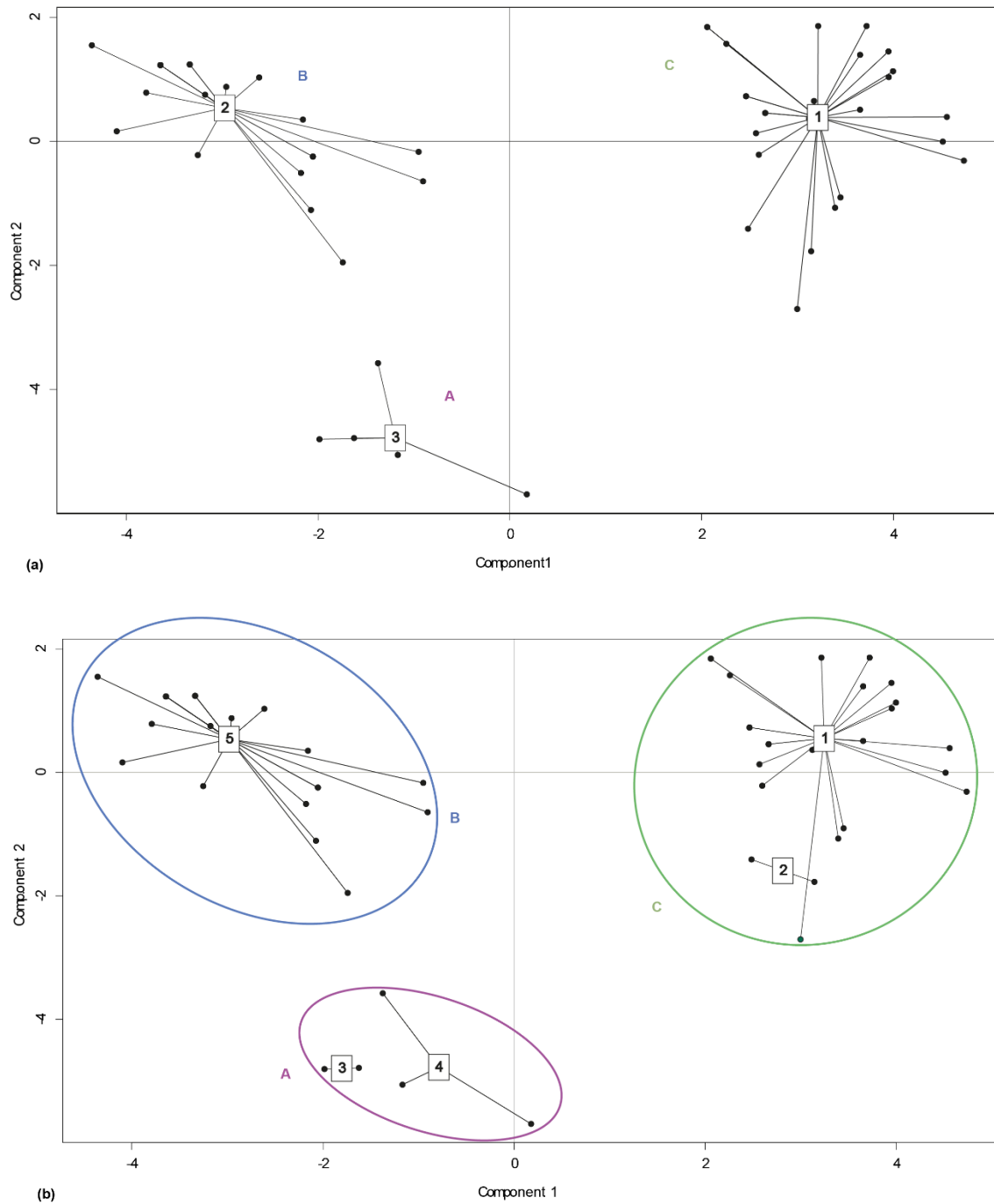


Figure 3: Multivariate component analyses representation of the variables “cortication” (a) and “siphon features” (b) in two component axes. Component axes 1 and 2 respectively explain 43.52 % and 14.71 % of the total variability (cumulative total: 58. 23%). In Fig 3.a, 1=absence of cortex, 2= complete cortex and 3= incomplete cortex. In Fig 3.b, 1=smooth siphons, 2 = smooth siphons with few protuberances in the transition zone between blade and stipe, 3= protuberances on blade siphons only in exposed areas, 4= protuberances all along blade siphons, 5= appendages. In Fig 3.a and b, A=incompletely corticated species (cortication= 3; siphon features= either 3 or 4), B= completely corticated species (cortication= 2, siphon features= 5), C= non-corticated species (cortication= 1, siphon feature= 1 or 2).

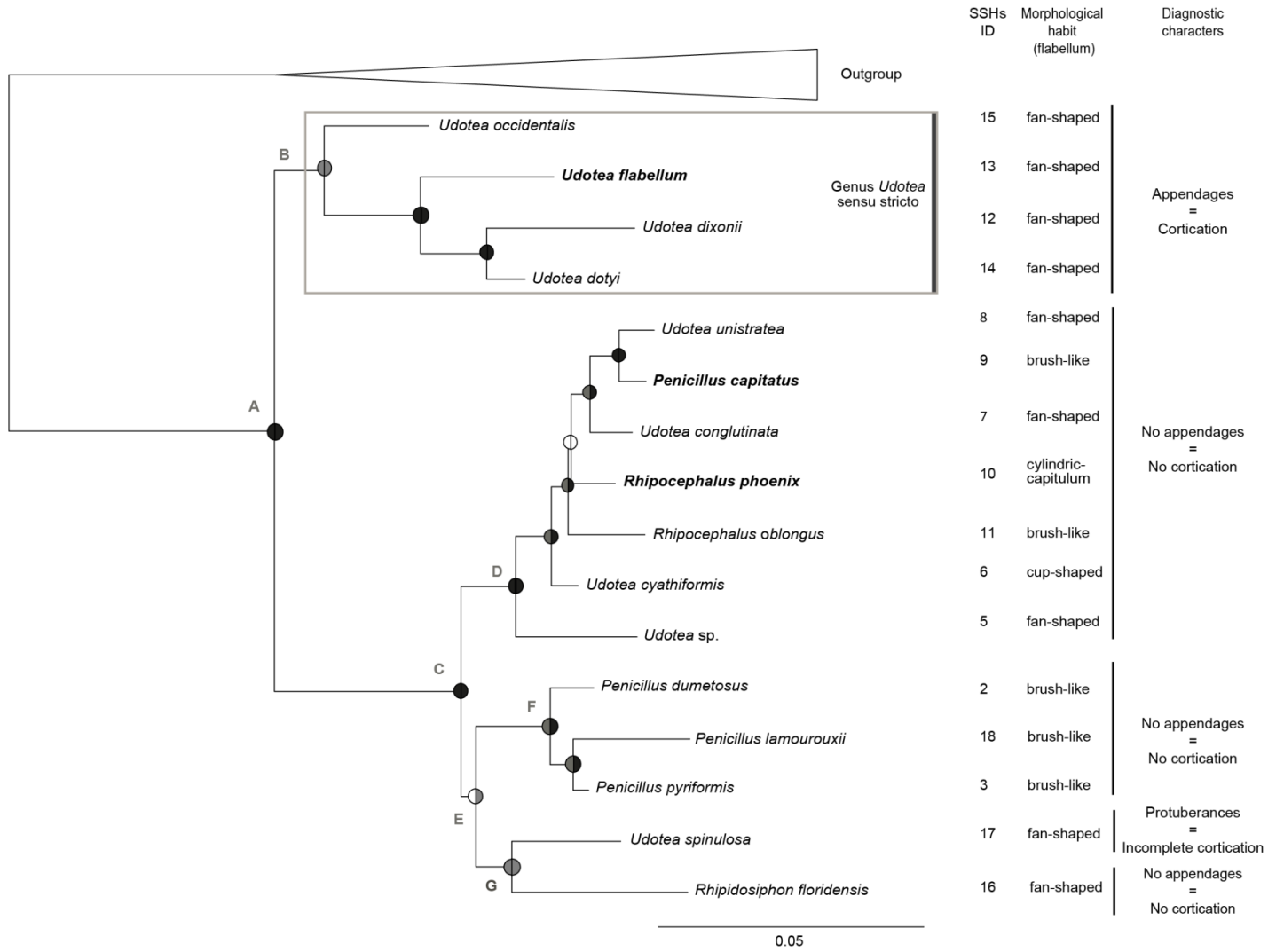


Figure 4: Maximum likelihood phylogenetic tree inferred from the multilocus matrix. At each node, circles represent ML bootstrap values (left) and Bayesian posterior probabilities (right): black circles indicate high support ($bs > 95$; $PP > 0.98$), dark grey circles represent moderate support ($95 > bs > 60$; $0.98 > PP > 0.70$), and white circles indicate absence of support ($bs < 60$ and $PP < 0.70$). Type-species are indicated in bold character. SSHs number ID (as per species delimitation analysis), morphological habit and diagnostic characters are indicated in the right columns. The genus *Udotea* s. s. is surrounded by the rectangle. Capital letters indicated the main nodes of the tree discussed in the text.

APPENDIX A: Supplementary material

Data A.1: List of morpho-anatomical variables used for the morphology-based delimitation analyses and associate code (if not applicable, value =0)

Data A.2: List of the PSHs obtained with the mPTP method applied on ML rooted tree for the *tufA* dataset

Data A.3: List of the PSHs obtained with the mPTP method applied on ML rooted tree for the *rbcl* dataset

Figure A. 1: (a) collecting sites for Udoteaceae specimens sampled during the PACOTILLES campaign (red circles) and DNA sequences provided by collaborators or available through Genbank (orange triangles). (b) Enlargement showing sampling sites in the Lesser Antilles

Figure A.2: Species delimitation results obtained using the five methods applied to the *rbcl* dataset. The tree is the maximum clade credibility tree from the BEAST analysis. Black bars indicate partitions retained as SSHs and grey bars indicate different partition schemes not retained.

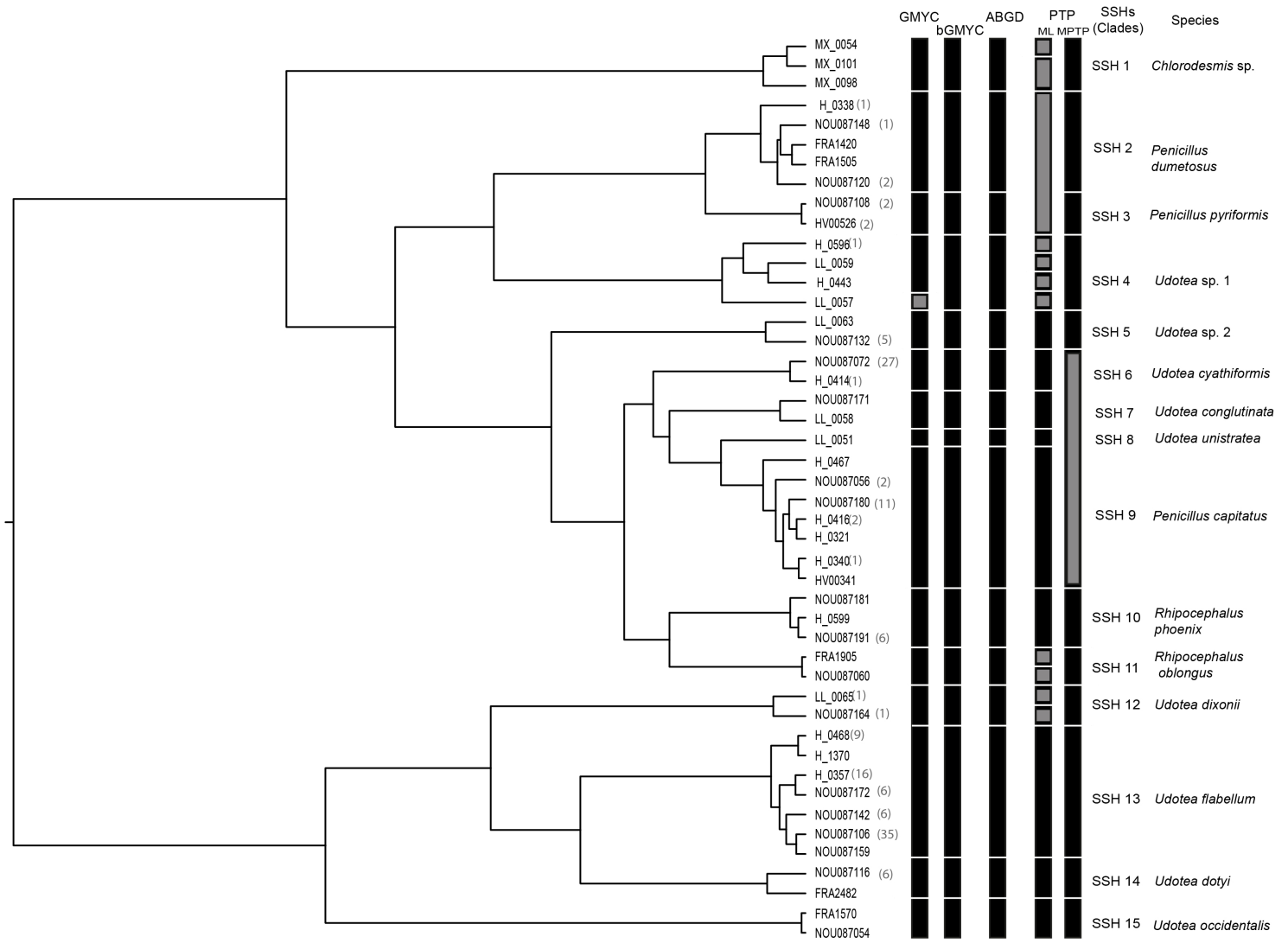
Figure A. 3: Results of the PCA analysis for all morpho-anatomical variables. These two components explain 58.23 % of the variability.

Table A. 1: Primers used for amplification of the *tufA*, *rbcl*, and 18S rDNA markers

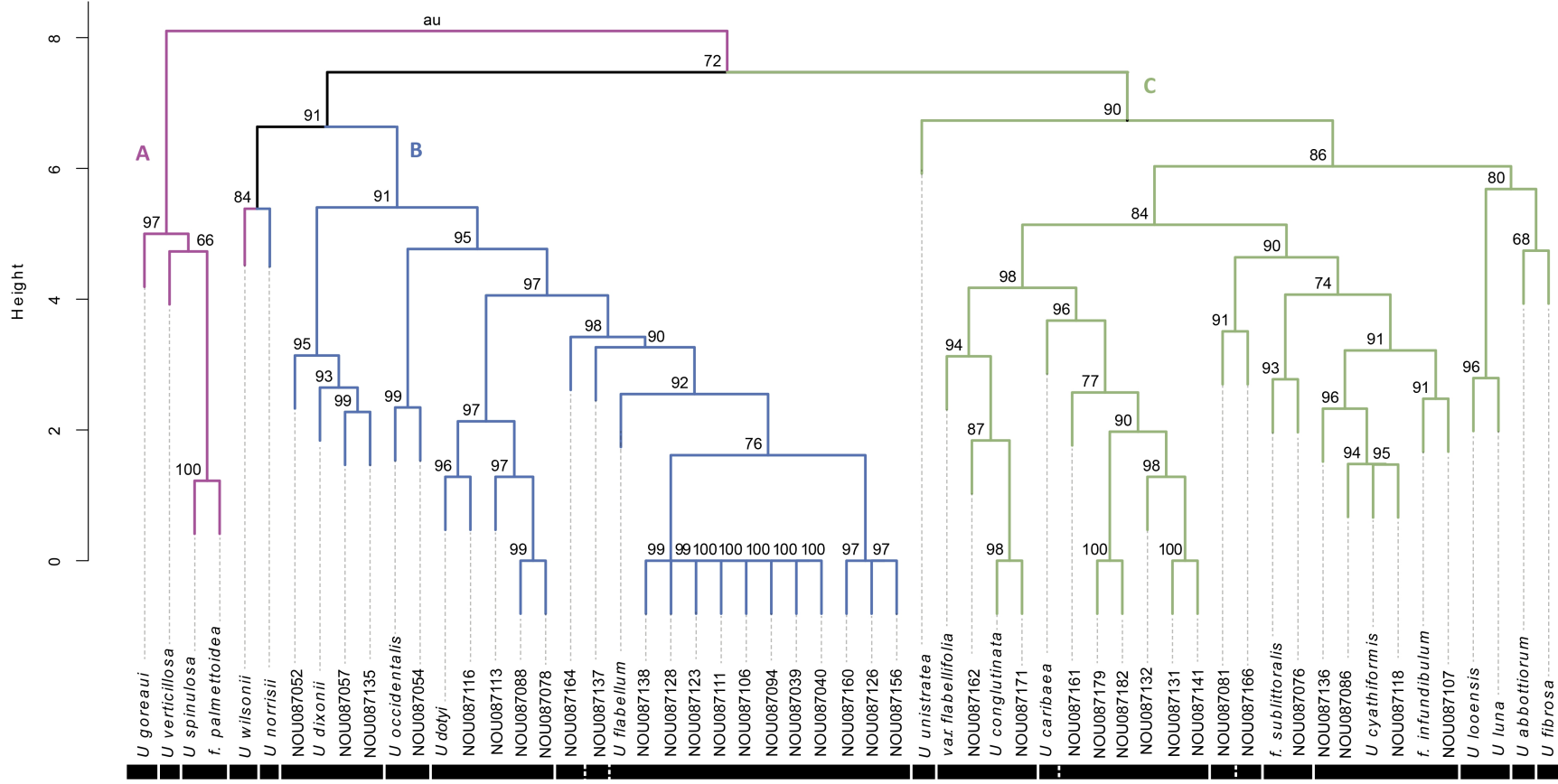
Table A.2: List of specimens with sample ID, species identification, location of sampling and Genbank accessions number (or BOLD sequence ID in grey for those not submitted)

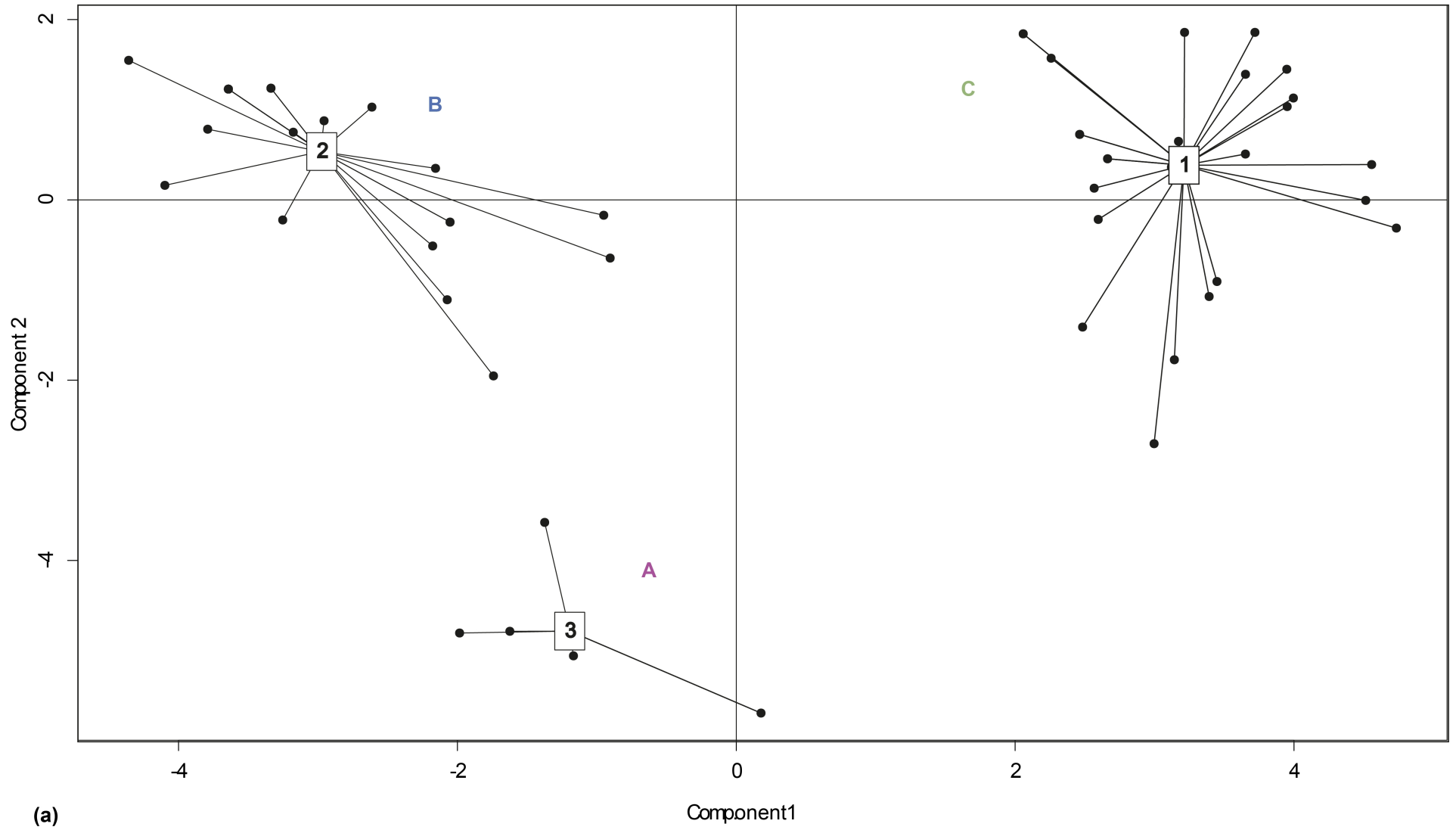
Table A.3: Table of the bootstraps values supporting the PSHs obtained with the PTP method on the *tufA* dataset.

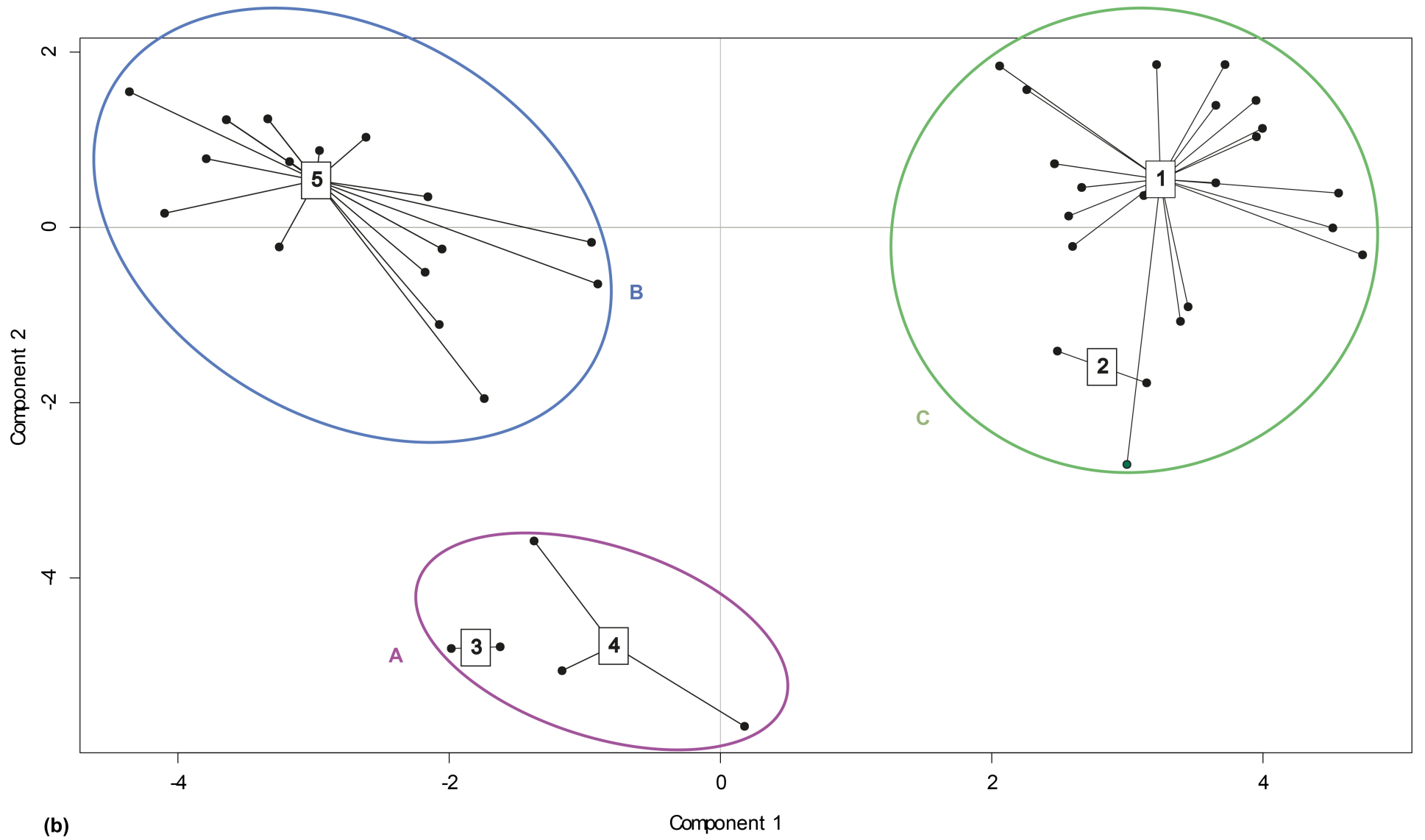
Table A.4: Table of the bootstraps values supporting the PSHs obtained with the PTP method on the *rbcl* dataset.



0.02







(b)

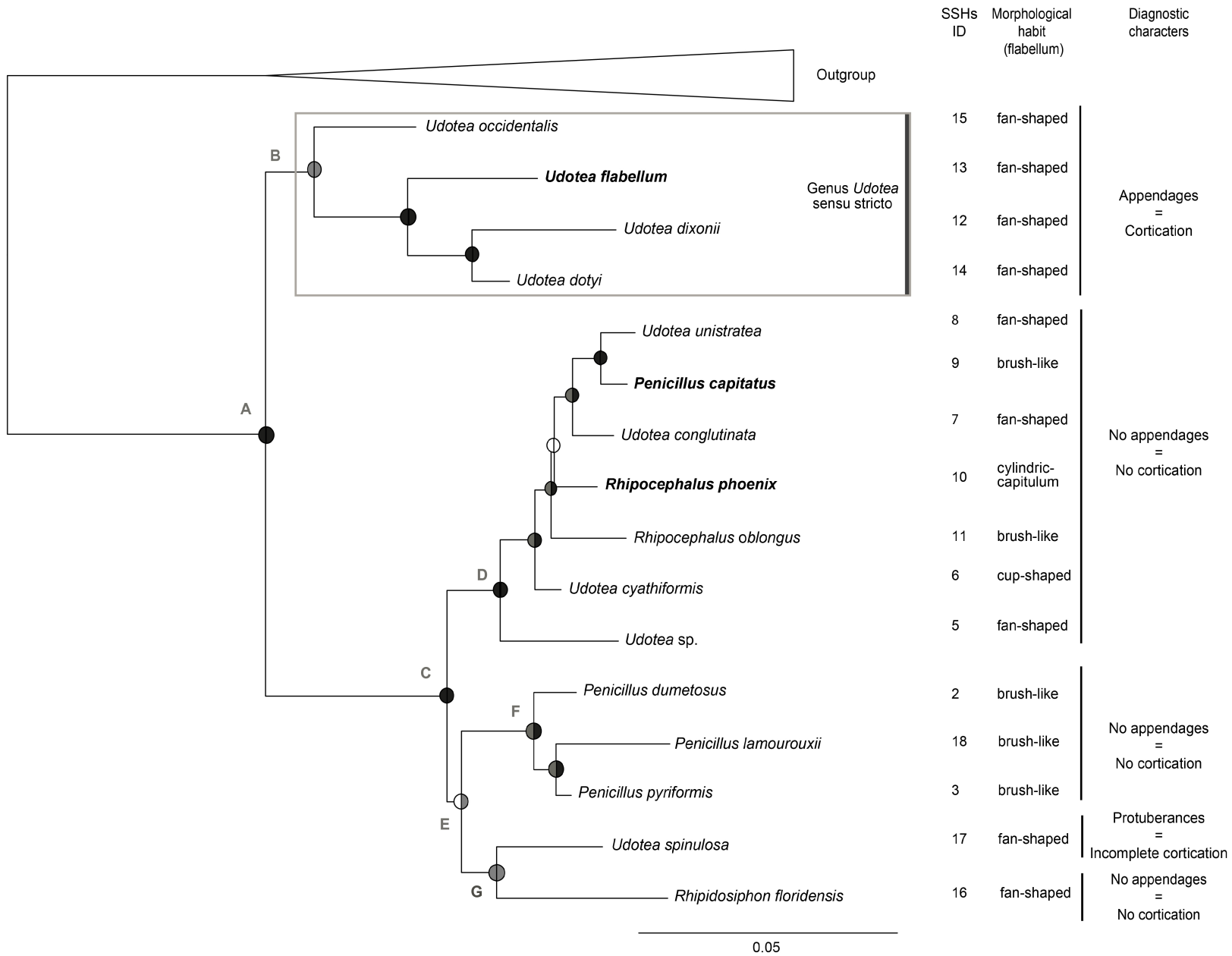


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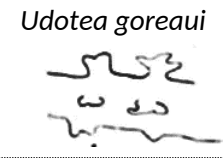
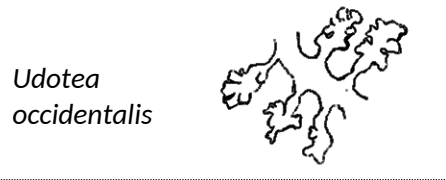
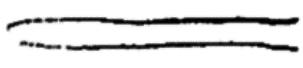
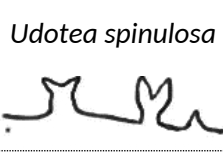

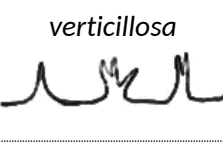
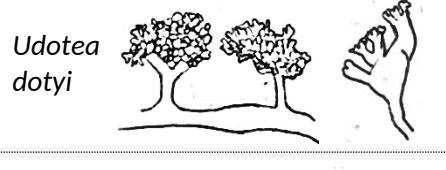
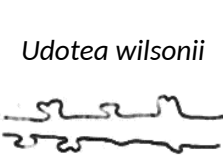


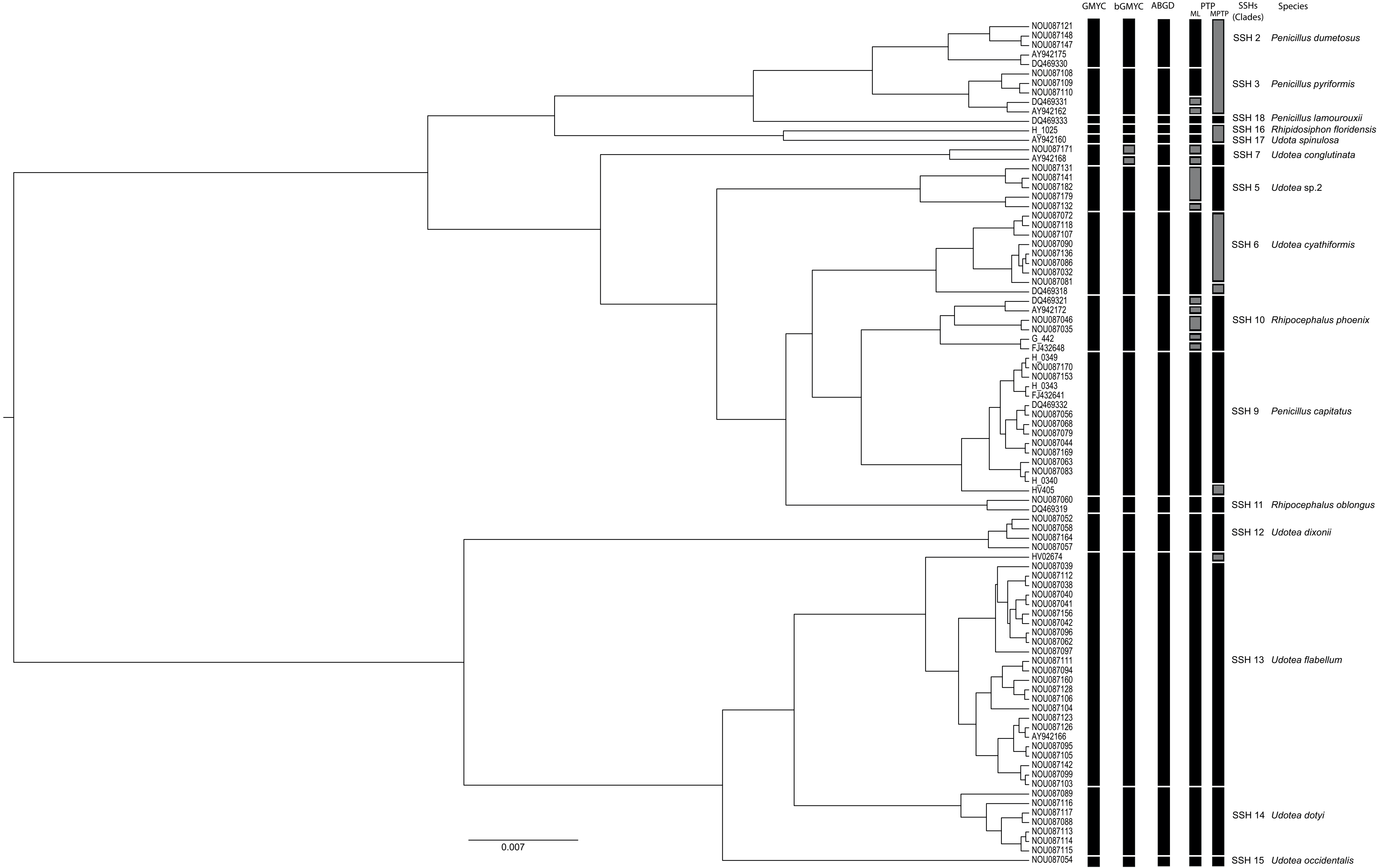
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	<i>Udotea spinulosa</i> 	<i>Udotea flabellum</i> 	
	<i>Udotea verticillosa</i> 	<i>Udotea dotyi</i> 	
	<i>Udotea wilsonii</i> 	<i>Udotea dixonii</i> 	
		<i>Udotea norrisii</i> 	

Table 1: Anatomical characters assessed for the identification of *Penicillus*, *Udotea* and *Rhipocephalus* species

<i>Genera</i>	Anatomical observations	
	Stipe filaments	Blade filaments
<i>Penicillus</i>	<ul style="list-style-type: none"> • Diameter • Appendage aspect (stalk, apices) 	<ul style="list-style-type: none"> • Diameter • Aspect (moniliform, tortuous or smooth) • Presence and narrowness of the constrictions above the dichotomies
<i>Rhipocephalus</i>	<ul style="list-style-type: none"> • Diameter • Appendage aspect (stalk, apices) 	<ul style="list-style-type: none"> • Diameter • Constant or degressive size of the filament from base to apex • Presence of dichotomies • Presence of constriction above dichotomies
<i>Udotea</i>	<ul style="list-style-type: none"> • Diameter • Appendage aspect (stalk, apices, dichotomous division) 	<ul style="list-style-type: none"> • Diameter • Aspect (moniliform, tortuous or smooth) • Presence of dichotomies • Presence of constriction above dichotomies • Presence of appendages and aspect (stalk, dichotomously division and apices)



0.007

Data A.2: List of the PSHs obtained with the mPTP method applied on ML rooted tree for the *tufA* dataset

Species 1:

NOU087164

LL_0065

Species 2:

NOU087106

H_0357

NOU087159

NOU087142

NOU087172

H_0468

H_1370

Species 3:

FRA2482

NOU087116

Species 4:

NOU087054

FRA1570

Species 5:

MX_0101

MX_0098

MX0_054

Species 6:

HV00526

NOU087108

H_0338

NOU087120

NOU087148

FRA1505

FRA1420

Species 7:

H_0596

LL_0059

H_0443

LL_0057

Species 8:

LL_0063

NOU087132

H_0414

NOU087072

NOU087171

LL_0058

NOU087060

FRA1905
NOU087181
H_0599
NOU087191
LL_0051
H_0467
NOU087056
H_0321
H_0340
H_0416
NOU087180
HV00341

Data A.3: List of the PSHs obtained with the mPTP method applied on ML rooted tree for the *rbcl* dataset

Species 1:

NOU087058
NOU087052
NOU087164
NOU087057

Species 2:

NOU087054

Species 3:

HV02674
NOU087042
NOU087038
NOU087112
NOU087096
NOU087105
NOU087123
AY942166
NOU087126
NOU087142
NOU087095
NOU087099
NOU087103
NOU087111
NOU087094
NOU087106
NOU087128
NOU087160
NOU087104
NOU087097
NOU087156
NOU087062
NOU087041
NOU087040
NOU087039

Species 4:

NOU087088
NOU087089
NOU087114
NOU087115
NOU087116
NOU087117
NOU087113

Species 5:

DQ469333
DQ469331
AY942162

NOU087108
NOU087109
NOU087110
DQ469330
AY942175
NOU087121
NOU087147
NOU087148
H_1025
AY942160
NOU087171
AY942168
NOU087131
NOU087179
NOU087141
NOU087182
NOU087132
NOU087107
NOU087118
NOU087032
NOU087081
NOU087136
NOU087086
NOU087090
NOU087072
DQ469318
NOU087046
NOU087035
HV404
DQ469321
AY942172
G_442
HV405
H0349
NOU087170
NOU087169
NOU087079
NOU087056
NOU087083
NOU087068
NOU087153
H_0340
H_0343
NOU087044
NOU087063
HV338
DQ469332
NOU087060
DQ469319

Data A.1: List of morpho-anatomical variables used for the morphology-based delimitation analyses and associate code (if not applicable, value =0)

Thallus habit (morphological characters)

BLADE

Bs: Shape of the blade: fan-shaped (1); cup-shaped (2)

Bd: Blade division: simple (1); divided or lobed (2); highly divided (3); several blades radiating from the axis (4)

Th: Thickness: unilayer (1); multilayer all along the blade (2); multilayer except at the margin (3); variable (4)

STIPE

J: Stipe-blade junction: sharp (1); pedicellate (2); continuous (3)

Std: Shape of the stipe: possibly branched (1); never branched (2)

Anatomical characters

BLADE

BSd: Siphons diameter: $\leq 50 \mu\text{m}$ (1); $50-100 \mu\text{m}$ (2) ; $\geq 100 \mu\text{m}$ (3)

BSa: Siphons aspect: monoliform at apices (1); torulose (2); strongly tortuous or contorted at apices (3); smooth (4)

BSdsp: Siphons disposition: parallel or subparallel (1); haphazard (2); subparallel to interwoven (3)

Bc: Constrictions above dichotomies: absent (1); even and elongated (2); uneven and elongated (3); even and well-marked (4); uneven and well-marked (5); occasional (6)

C: Cortication: absent (1); complete cortex (2); incomplete cortex (3)

CS: Calcareous sheath: not porous (1); porous (2)

BA: Siphons features: smooth (1); smooth with outgrowths on basal siphons from the blade-stipe transition zone (2); scattered protuberances (3); numerous protuberances all along the siphon (4); appendages (5)

BAnb: Appendages or protuberances quantity: numerous (1); sparse (2)

BAAd: Appendages or protuberance disposition: Scattered (1); in ranks of (2); in ranks of 4 (3); opposite to haphazard (4); only on one side (5)

BAa: Appendages or protuberances attachment: sessile or sessile (1); peltate or pedicellate (2); stalk (3), variable (4)

BAP: Aspect of the appendages or protuberances: lobes (1); finger-like (2); very short dichotomies (3); spines or papillae (4); both lobes and short dichotomies (5)

BT: Tips aspect of the appendages: knobby (1); rounded (2); acute (3); swollen rounded or flattened (4); swollen or acute (5)

STIPE

SSd: Siphons diameter: $\leq 50 \mu\text{m}$ (1); $]50-100[\mu\text{m}$ (2) ; $[100-150[\mu\text{m}$ (3); $\geq 150 \mu\text{m}$ (4)

Sc: Constrictions: absent (1); present and equal (2); present and unequal (3); occasional (4)

SAa: Appendages attachment: short stalk (1); long stalk (2); variable (3)

SAP: Appendages aspect: highly dichotomously divided (1); finger-like/few branches (2)

ST: Tips aspect: acute (1); rounded (2); stubby (3); swollen rounded or flattened (4); acute or rounded (5)

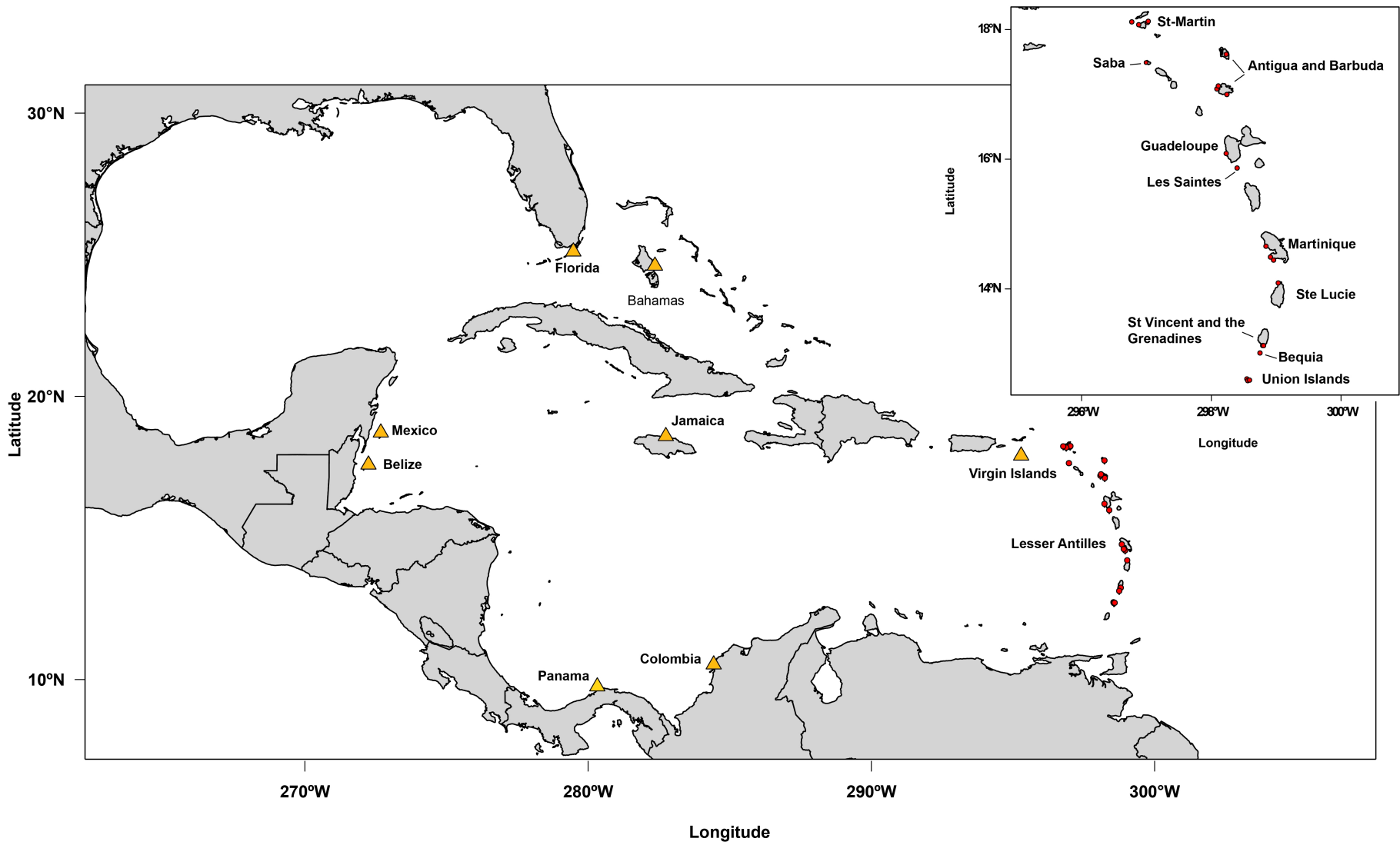


Table A. 1: Primers used for amplification of the *tufA*, *rbcL*, and 18S rDNA markers

Genes	Primers names	primer sequence (5'-3')	Direction	References
<i>tufA</i>	tufA-GF4	GGNGCNGCNCAAATGGAYGG	Forward	Saunders and Kucera, 2010
	tufAR	CCTTCNCGAATMGCRAAWCGC	Reverse	
	HtufAF	ATGATWACNGGHGCNGCWCAAATGG	Forward	Händeler et al., 2010
	HtufAR	TTGTTCKAACATAAAAATTGWGGTC	Reverse	
<i>rbcL</i>	rbcL-7F	CCAMAAACWGAAACWAAAGC	Forward	Lam and Zechman, 2006 and Verbruggen et al., 2009
	rbcL-1391R	TCTTTCCAAACTTCACAAGC	Reverse	
	rbcL-712F	CATTAYTTAAATGCWACWGC	Forward	Verbruggen et al., 2009
	rbcL-791 R	GGNAYACCNAAWTCTTTIGC	Reverse	
18S rDNA	TW3F	GCAAGTCTGGTGCCAGCATCT	Forward	Kooistra, 2002 and Verbruggen et al., 2009
	SS09HF	GGTGAAATTCTTGGATTTRYGRAAGAC	Forward	
	SS11HR	CCTTTAAGTTTCAGCCTTGCGACC	Reverse	
	SS17HR	CCTTGTTACGACTTCTCCTTCCTC	Reverse	