Toward an inordinate fondness for stars, beetles and Lobophora? Species diversity of the genus Lobophora (Dictyotales, Phaeophyceae) in New Caledonia

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TOWARD AN INORDINATE FONDNESS FOR STARS, BEETLES AND *LOBOPHORA*? SPECIES DIVERSITY OF THE GENUS *LOBOPHORA* (DICTYOTALES, PHAEOPHYCEAE) IN NEW CALEDONIA[1]

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Abstract

Until the recent use of molecular markers, species diversity of *Lobophora*, an ecologically important brown algal genus with a worldwide distribution in temperate and tropical seas, has been critically underestimated. Using a DNA-based taxonomic approach, we re-examined diversity of the genus from New Caledonia in the Southwest Pacific Ocean. First, species were delineated using GMYC-based and barcoding gap approaches applied to a mitochondrial *cox*3 dataset. Results were subsequently confirmed using chloroplast *psb*A and *rbc*L datasets. Species delimitation analyses agreed well across markers and delimitation algorithms, with the barcoding gap approach being slightly more conservative. Analyses of the *cox*3 dataset resulted in 31 to 39 molecular operational taxonomic units, four of which are previously described species (*L. asiatica , L. crassa, L. nigrescens* s.l.*, L. pachyventera*). Of the remaining MOTUs for which we obtained a representative number of sequences and results are corroborated across analyses and genes, we describe ten species *de novo*: *L. abaculusa, L. abscondita*, *L. densa*, *L. dimorpha*, *L. gibbera*, *L. hederacea*, *L. monticola*, *L. petila*, *L. rosacea*, and *L. undulata*. Our study presents a excellent case of how a traditional morphology-based taxonomy fails to provide accurate estimates of algal diversity. Furthermore, the level of *Lobophora* diversity unveiled from a single locality in the Pacific Ocean raises important questions with respect to the global diversity of the genus, the distributions and range sizes of the individual species, as well as the mechanisms facilitating co-existence.

Key index words: *Lobophora*, GMYC, ABGD, species delimitation, New Caledonia, new species, phylogeny, taxonomy

45

***Abbreviations*:** ABGD, automated barcoding gap discovery; AIC, Akaike information criterion; ANOVA, analysis of variance; BI, Bayesian inference; bGMYC, Bayesian implementation of the general mixed Yule coalescent; CTAB, cetyltrimethyl ammonium bromide; GMYC: general mixed Yule coalescent; GTR, generalized time reversible; MCMC: Markov chain of Monte Carlo; ML: maximum likelihood; MOTU: molecular operational taxonomic unit; Tukey HSD: Tukey Honestly Significant Difference.

Introduction

Contrary to substantial historical disagreement on the generic classification of the genus *Lobophora* J.Agardh (J.V.Lamouroux 1809, C.Agardh 1817, J.Agardh 1894, Papenfuss 1943, Womersley 1967), species-level taxonomy has been remarkably stable. Traditionally only three *Lobophora* species were recognized, with *L. variegata* (J.V.Lamouroux) Womersley ex E.C.Oliveira being by far the most commonly reported species. Literature data make it seem that *L. variegata* is widely distributed in temperate to tropical parts of the Atlantic (incl. Mediterranean Sea), Indian and Pacific Ocean. The other two species *L. papenfussii* (W.R.Taylor) Farghaly and *L. dichotoma* (R.H.Simons) P.C.Silva were only sporadically reported from the Indo-Pacific and South Africa respectively. From 2000 until 2012, three more species were described (*L. minima* V.Krishnamurthy and M.Baluswami (2000), *L. indica* V.Krishnamurthy and M.Baluswami (2000) and *L. rickeri* Kraft (2009)), based on morphological criteria only.

From a molecular phylogenetic perspective *Lobophora* had not received much attention (but see Hoshina et al. 2004, Phillips et al. 2008, Bittner et al. 2008) until a recent study of Sun et al. (2012). The latter authors recognized nine major *Lobophora* clades based on chloroplast *rbc*L and mitochondrial *cox*3 gene sequences, four of which were formally described as new species (*i.e. L. asiatica* Z.Sun, Ji.Tanaka and H.Kawai*, L. crassa* Z.Sun, P.-E.Lim and H.Kawai*, L. pachyventera* Z.Sun, P.-E.Lim, Tanaka and H.Kawai*, L. australis* Z.Sun, Gurgel and H.Kawai). In total, 10 species are currently accepted taxonomically (Guiry and Guiry, 2013).

Despite the ecological importance of *Lobophora* in seaweed-coral-grazing interactions and competition (De Ruyter van Steveninck and Breeman 1987a,b, De Ruyter van Steveninck et al. 1988a,b,c, Coen and Tanner 1989, Diaz-Pulido et al. 2009, Rasher and Hay 2010, Anthony et al.

77 2011, Slattery and Lesser 2013), the species diversity of the genus remains largely unaddressed.
78 Here we study the diversity of *Lobophora* in New Caledonia. New Caledonia is located just
79 south of the coral triangle, recognized as the global center of marine biodiversity, and displays
80 tropical to subtropical-temperate conditions. The *Lobophora* flora has been comprehensively
81 sampled over the last decades from various regions and the large amount of material revealed a
82 large morphological diversity associated to the ecological variation justifying the present study.
83 The paper of Sun et al. (2012) provided two important insights about the genus *Lobophora*, (1)
84 the existence of a rich and yet to be discovered diversity and (2) the occurrence of cryptic
85 diversity lacking distinctive morphological features between taxa.
86 Decisions on species concepts as well as the practical criteria to delimit species represent critical
87 aspects for studies aiming to elucidate species level diversity (*e.g.* Harrison 1998, Agapow
88 2004). For algae it has long been recognized that diversity is often inadequately reflected in the
89 organism's morphology. It is therefore not surprising that, coinciding with a growing ease to
90 obtain molecular data, the latter have become the standard for delimiting algal species (see
91 Alverson 2008; De Clerck et al. 2013; Leliaert et al. 2014). Accompanying a growing
92 dependency on DNA sequence data in biodiversity assessment, a variety of approaches and
93 algorithms have been proposed to detect discontinuities in genetic variation representative for
94 species boundaries (*e.g.* Wiens and Penkrot 2002, Sites and Marshall 2004, Carstens et al. 2013).
95 Since, species delimitation may be influenced by the gene information content as well as the
96 species delimitation method, we test species boundaries in *Lobophora* using three species
97 delimitation methods, a General Mixed Yule Coalescent (GMYC) model (Pons et al. 2006,
98 Fujisawa and Barraclough 2013), the Bayesian implementation of the GMYC model (Reid and
99 Carstens 2012) and an Automated Barcoding Gap Discovery method (ABGD) (Puillandre et al.

100 2011). The combination of several molecular methods for species delimitation is becoming a
101 reference to detect species boundaries and have been used in different taxonomical groups
102 (Jörger et al. 2012 for sea slugs; Kekkoken and Hebert 2014 for moths; Cornils and Held 2014
103 for copepods; Alò et al. 2013 for fishes). To our knowledge it is the first time that such a
104 combination is used for algae species delimitation.
105 Species delimitation is in the first place carried out using a mitochondrial *cox*3 dataset for which
106 we had the most complete taxon sampling. To investigate up to which extent results were
107 influenced by marker choice, analyses were repeated for chloroplast *rbc*L and *psb*A datasets,
108 which contained less sequences per taxon compared to the *cox*3 dataset. Subsequently, we
109 studied the morphology and ecology of the New Caledonian specimens to determine up to which
110 extent the DNA-based species are morphologically and ecologically diverged.
111
112 Materials and Methods
113 *Sampling*
114 *Lobophora* specimens were collected from 41 locations in New Caledonia (FIG. 1). Most of New
115 Caledonia was sampled, except for the remote Entrecasteaux reefs. Sampling sites included the
116 southwest lagoon of Grande Terre (collections between 2004 and 2013), Isle of Pines (BIODIP,
117 November 2005), the Loyalty Islands (BSM-Loyauté, March-April 2005), La Côte Oubliée
118 (CORALCAL1, March 2007), the Chesterfield-Bellona-Bampton area (CORALCAL2, July
119 2008), Le Grand Lagon Nord (CORALCAL3, February 2009), and different sites along the north
120 west and north east coasts of Grande Terre (CORALCAL4, November-December 2012).
121 Sampling was carried out mainly by SCUBA from 3 down to 90m deep or by snorkeling and reef
122 walking. The specimens were readily stored in a cooler and desiccated in silica gel for

subsequent DNA extraction once at the laboratory. Specimens were dried and mounted on herbarium sheets and deposited at the IRD Herbarium of Nouméa (New Caledonia, IRD-NOU). For the earliest collections, dry Herbarium specimens were used as DNA source. The New Caledonia samples were complemented with a few collections from Papua New Guinea (Madang 2012) and the Maldive Islands (2011). The origin of the specimens and accession numbers are detailed in TABLE S1.

DNA extraction, amplification, sequencing and phylogenetic analyses

Total genomic DNA was extracted from 235 *Lobophora* samples, 228 from New Caledonia, 5 from Papua New Guinea and 2 from the Maldive Islands using a CTAB-extraction method (De Clerck et al. 2006). Genomic DNA was subsequently purified with a Wizard® DNA Clean-Up System (Promega Inc., Madison, WI, USA) following the manufacturer's instructions. Sequences were generated from one mitochondrial gene (*cox*3), two chloroplast genes (*psb*A, *rbc*L) and the 5'-end of the nuclear encoded large subunit rDNA (LSU, ca. 1200 bp). PCR and sequencing conditions are detailed in TABLE S2. LSU sequences were not tested for species delimitation because of the low number of sequences obtained, but were integrated in the concatenated alignment to generate a species tree with improved resolution. In addition to the sequences generated in the present study, 25 *cox*3, 4 *psb*A, 33 *rbc*L and 6 LSU *Lobophora* sequences from GenBank were added to the alignments (TABLE S1). Sequences were aligned using MUSCLE implemented in eBioX 1.5.1 (www.ebioinformatics.org). Ambiguously aligned regions in the LSU alignment were removed by eye.

Species delimitation

146 Following exploratory ML and Bayesian analyses (results available upon request), ultrametric
147 gene trees were constructed using Bayesian analyses in BEAST v1.7.5 (Drummond et al. 2012)
148 for the *cox*3, *rbc*L and *psb*A alignments. A GTR+G substitution model was identified as the best-
149 fitting model for each individual gene, based on the Akaike Information Criterion (AIC) using
150 jModelTest 2 (Darriba et al. 2012). BEAST analyses were run under a strict molecular clock in
151 combination with a Constant Coalescent tree prior. Other priors were set to default. In order to
152 check for convergence of the MCMC chains, we performed two independent runs for 10^7
153 generations each, starting from random trees and sampling every 10^4 generations. MCMC output
154 files of the independent runs were inspected in Tracer 1.5 (Rambaut and Drummond 2009) for
155 acceptable effective sample sizes (ESS > 200). A burn-in was applied once log-likelihood values
156 had stabilized. Maximum clade credibility trees and posterior probability for the nodes were
157 calculated using the postburnin trees using TreeAnnotator 1.6.2 (included in the BEAST
158 package). All tree searches were conducted on the Cipres web portal (Miller et al. 2010).
159 We used a Maximum Likelihood (GMYC) as well as a Bayesian Implementation (bGMYC) of
160 the GMYC model (Pons et al. 2006; Reid and Carstens 2012). Both methods are able to
161 discriminate between population and speciation patterns on a given ultrametric tree. GMYC
162 analyses under a single-threshold were conducted in R (R Core Team, 2014) using the package
163 "Splits". The bGMYC model was performed using "bGMYC" (Reid and Carstens 2012) in R
164 using a subsample of 100 trees from the posterior distribution of BEAST as suggested by the
165 authors. Markov chain Monte Carlo (MCMC) chains were run for each tree for 10,000
166 generations with a burn-in comprising the first 1,000 generations once the log-likelihood values
167 had stabilized, and sampling every 100 generations.

168 Automatic Barcode Gap Discovery (ABGD, Puillandre et al. 2012) is an exploratory tool based
169 on pairwise distances to detect automatically significant difference in intra and inter specific
170 variation (*i.e.* barcoding gap), without an *a priori* species hypothesis. These analyses were
171 performed on the abgd website (wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html. Accessed
172 2013 October 12) selecting default parameters except for the relative gap width (X) which was
173 set to 1 and the number of steps which was set to 100. The distance matrix was build under a
174 K2P model.
175 Species boundaries were subsequently defined based on the congruence of the three methods and
176 are detailed in the discussion.
177 *Species tree inference*
178 Maximum likelihood (ML) and Bayesian inference (BI) phylogenetic species trees were
179 generated from a concatenated alignment including *cox*3 (610 bp), *psb*A (919 bp), *rbc*L (1360
180 bp) and LSU rDNA (1361 bp) genes, partitioned by gene and codon position. The concatenated
181 alignment contained a single representative per Molecular Operational Taxonomic Unit (MOTU)
182 resulting from the species delineation analyses of the *rbc*L dataset. The matrix was 70% filled at
183 the MOTU level. A selection of *Zonaria* C.Agardh (Dictyotales, Phaeophycea), *Padina* Adanson
184 (Dictyotales, Phaeophycea) and *Dictyota* J.V.Lamouroux (Dictyotales, Phaeophycea) species
185 were used as outgroup taxa (cf. TABLE S1). ML analyses were conducted using RAxML under
186 a GTR+CAT model (Stamatakis 2006). The robustness of the resulting phylogenies was tested
187 using 1000 replicates of a rapid bootstrap heuristic (Stamatakis et al. 2008). BI, using MrBayes
188 v3.2.2 (Ronquist and Huelsenbeck 2003), initiated with a random starting tree and ran four
189 chains of MCMC iterations simultaneously for 100 million generations. The first 100,000 (25%)
190 trees sampled were discarded as burn-in, based on the stationarity of ln*L* as assessed using Tracer

191 version 1.5 (Rambaut and Drummond 2009). A consensus topology and posterior probability
192 values were calculated from the remaining trees.
193
194 *Morphological and ecological analyses*
195 Morphological observations of *Lobophora* species included analyses of the external and internal
196 (anatomy) structure of the specimens. Based on our field observations we distinguished the
197 occurrence of seven main growth forms, namely (1) stipitate, (2) fasciculate, (3) conk-like, (4)
198 decumbent, (5) anastomosing, (6) procumbent and (7) crustose as illustrated and defined in FIG.
199 2. For the internal morphology, longitudinal and transverse sections were made of the basal,
200 middle and distal portions of the thallus using a medical freezing portable microtome
201 (Labonord®). Photographs of the sections were taken with a digital camera (Olympus Camedia
202 C-5050 5.0 Megapixel, Tokyo, Japan) attached to a compound microscope (Olympus BH-2,
203 Tokyo, Japan). The number and size of the cortical (dorsal and ventral) and medulla cells of the
204 basal, middle and distal portions of the thallus were measured as shown in FIG. 3, which resulted
205 in the measurements of 9 anatomical traits (*i.e.* number of dorsal and ventral cells; total number
206 of cells; thallus thickness; dorsal, medullar and ventral heights; medullar width and length). The
207 surface of the thallus with rhizoids was defined as the ventral surface. A total of 285 specimens,
208 from one to 15 specimens per species, were examined for morphological analyses. Every
209 specimen studied morphologically has been sequenced for at least the *cox*3 marker. A few
210 sequences which were too short were not included in the molecular analyses. Descriptive
211 statistics were generated for the anatomical traits and correlations between them were tested to
212 select independent traits for subsequent univariate analyses. Mean anatomical traits were tested
213 for equality by a one-way ANOVA and post-ANOVA Tukey Honestly Significant Difference

(HSD) tests. The data were tested for normality and homogeneity of variances by means of a Shapiro-Wilk test of normality and the Bartlett test of the homogeneity of variances. The thickness data were log-transformed prior to analysis, to meet assumptions of normality and homogeneity of variance. All analyses were conducted using R. Ecologically, we identified three major substratum preferences in the field specific to some groups of species: (1) niched among or growing on live corals, (2) growing at the base of live corals, on dead corals, coral rubbles or bedrock and (3) growing niched among *Sargassum* beds.

Results

Species delimitation

Species delimitation based on the *cox*3 alignment (610 bp x 210 sequences) using GMYC under a single threshold resulted in an estimate of 37 for MOTUs, with a confidence interval of 36-49 (FIG. 4). The number of specimens per MOTU ranged from 1 (singletons) to 45 with an average of 6.5. bGMYC analysis of posterior probabilities of conspecificity within *cox*3 *Lobophora* clusters was high ($P > 0.9$) and resulted in a species delimitation which was marginally less conservative than GMYC, differing in 2 cases only (FIG. 4): IRD10187 was resolved as a singleton (prob. 0.59), d271 and d6625 were resolved as a separate cluster (prob. 0.648). The ABGD approach is slightly more conservative, grouping four MOTUs that were split in both GMYC analyses (FIG. 4).

Species delimitation analyses were repeated for *rbc*L (1345 bp x 139 sequences) and *psb*A (919 bp x 88 sequences) datasets to investigate if the *cox*3 results were stable across genes. In all analyses the likelihood of the GMYC model was significantly higher ($p < 0.001$) than that of the null model of uniform coalescent branching rates. GMYC analyses of *rbc*L data yielded (40-) 47

(-54) MOTUs while the *psb*A data resulted in (17-) 19 (-34) MOTUs (TABLE 1). Contrary to the *cox*3 dataset, no incongruence between the various delimitation methods was detected. Unequal sampling across markers complicates a detailed comparison of results from different markers, but even without a fully congruent sampling it was clear that the outcome of the analyses was stable across genes (TABLE S3). Six MOTUs from the *cox*3 ABGD analysis were subdivided in less inconclusive units in the *rbc*L dataset. All but one of the *cox*3 bGMYC MOTUs on the other hand were confirmed in the *rbc*L dataset. Data from the *psb*A dataset are less informative because of the high number of missing MOTUs (47%), but of the *cox*3 ABGD MOTUs present 2 are subdivided and one is merged with another MOTU. Similarly, 2 *cox*3 bGMYC MOTUs are merged. In addition, inclusion of Genbank accessions in the *rbc*L dataset yielded 9 additional MOTUs, which were not represented in either the *cox*3 or *psb*A dataset. This resulted in the *rbc*L gene alignment being the most diverse in terms of MOTUs, but with a significantly higher number of singletons than *cox*3.

Morphological and ecological characters

The morphology and ecology of the specimens from New Caledonia were studied to determine up to which extent the MOTUs are morphologically and ecologically diverged. For practical reasons we introduce names of newly described species already in the sections below. Results and interpretations of correlation analyses between the nine anatomical characters measured are given in the supplementary text (TABLE S4). Boxplots were used to show inter- and intra-specific variation of six anatomical traits (thallus thickness; dorsal and ventral height; medulla height, width and length) (FIG. 5). Anatomical characters related to cell height differed significantly among species as well. On the other hand cell length and width displayed some

variation but were overall less diagnostic. Among the three independent anatomical traits (*i.e.* thallus thickness, medulla width and length), the thallus thickness presented the most significant interspecific variability and was therefore retained as the only variable for the ANOVA analysis. The thallus thickness ranged from an average of 57 µm for the thinnest species (*L. petila*) to 407 µm for the thickest species (*L. densa*). A continuous grade from these two extreme values was observed and the thickness of several species overlapped. The amount of intraspecific variation differed, with the thicker species presenting a greater variability. A one-way ANOVA analysis (TABLE S4) revealed statistically significant differences and subsequent post-hoc analyses (Tuckey HSD) (TABLE S5) confirmed significant difference between the species thallus thickness means. Seven species presented unique means and distribution (*L. densa, L. crassa, L. gibbera, L. hederacea, L. monticola, L. pachyventera* and *L. petila*) and three groups of species exhibited neighboring mean values with comparable variances (FIG. S1). Consequently, thallus thickness may serve to identify seven New Caledonian species but for some groups of species does not suffice to go down to the species level delineated with the phylogenetic approaches. However, for those 3 groups with similar thickness, external morphology and ecology allow species differentiation (see below).

Species phylogeny

ML and BI analyses of the concatenated alignment (*cox*3 + *rbc*L + *psb*A + LSU) including every MOTU discovered in the species delimitation analyses, yielded similar tree topologies except for the relationships between the MOTUs 29 to 32, and the MOTUs 45 to 47. Results are presented using the BEAST ultrametric tree topology (FIG. 6). The 4-genes analyses resulted in a fairly well-resolved phylogeny with moderate to strong support for most nodes. The phylogenetic tree

283 revealed 6 well-supported lineages (defined as a sequence of species or MOTUs; Lineage A-F)
284 (FIG. 6). However, the position of the MOTU 46 from Guadeloupe, for which only the *rbc*L
285 sequence is available, is incongruent between the trees. In the BEAST and ML trees MOTU 46 is
286 part of the Lineage A (FIG. 6 and S2), while it comes outside of the Lineage A, in the most basal
287 position, in the Bayesian tree (FIG. S3). This inconsistency may be resolved by acquiring extra
288 sequences for the missing markers, and for the time being we will consider it as part of the
289 Lineage A.
290
291 Discussion
292 *Species diversity and taxonomy*
293 In this study we aimed to characterize the diversity of the genus *Lobophora* in New Caledonia in
294 the South West Pacific Ocean and subsequently address the evolutionary relationships of the
295 New Caledonian representatives. Thereto, we applied the most comprehensive sampling of the
296 genus to date. Although expecting some levels of cryptic or pseudocryptic diversity, much to our
297 initial astonishment *cox*3 species delimitation analyses yielded between 31, 37 and 39 MOTUs
298 based on ABGD, GMYC and bGMYC analyses, respectively. Both GMYC-based methods were
299 highly congruent. The bGMYC analyses segregated one specimen (IRD10187) from *Lobophora*
300 *crassa*2. Likewise, d271 and d6625 were segregated from *L. nigrescens* s.l. Both results,
301 however, were only moderately supported in the bGMYC analysis, with posterior probabilities of
302 0.591 and 0.648 respectively. The barcoding gap method yielded a more conservative estimate,
303 but most discrepancies were limited to the *L. crassa* and *L. pachyventera* complexes as defined
304 by Sun et al. (2012) and discussed below.
305 Subsequent analyses of *rbc*L and *psb*A dataset were highly congruent with the GMYC and

306 bGMYC results and indicated that the ABGD estimate of the *cox*3 dataset is likely somewhat
307 over-conservative (TABLE 2 and TABLE S6). Possibly the small sample size of some MOTUs
308 may result in larger units as identified by the barcoding gap approach (Jorger et al. 2012,
309 Puillandre et al. 2012). We identified one case in which the *cox*3 GMYC analyses were too
310 conservative (SAP109520) compared to *rbc*L results, and one case in which they were too liberal
311 (IRD10187). In both situation a single specimens was either added to or segregated from a
312 MOTU.
313 Our analyses disclosed the occurrence of 29 MOTUs in New Caledonian. These results confirm
314 findings by Sun et al. (2012) of undescribed species diversity in *Lobophora.* Species boundaries
315 as defined by Sun et al. (2012) of *L. asiatica, L. nigrescens* sensu Sun et al. (2012) (subsequently
316 referred to as *L. nigrescens* s.l.) and *L. australis* are mirrored by our species delimitation.
317 However, their species delineation appeared to be more conservative for *L. crassa* and *L.*
318 *pachyventera*. GMYC and bGMYC analyses split the *L. crassa* and *L. pachyventera* complexes
319 into five and four MOTUs respectively for *cox*3 (FIG. 4). In the *L. crassa* complex the New
320 Caledonian specimens were resolved as separate MOTUs, *L. crassa*2, *L. crassa*4 and *L. crassa*5.
321 Likewise, in the *L. pachyventera* complex the New Caledonian specimens were resolved as a
322 separate MOTU, *L. pachyventera*2. However, it should be noticed that the *cox*3 ABGD results
323 group the *L. crassa* MOTUs and *L. pachyventera1*, *L. pachyventera2* and *L. pachyventera3* in
324 two clusters only. Four of the New Caledonian MOTUs, were assigned to existing species or
325 species complexes (*Lobophora crassa*, *Lobophora asiatica, Lobophora pachyventera* and *L.*
326 *nigrescens* s.l.). In addition, none of our samples matched the descriptions of the four *Lobophora*
327 species for which no molecular data are available (*i.e. L. variegata, L. dichotoma*, *L. rickeri*, *L.*
328 *papenfussii*). The remaining MOTUs could therefore qualify as putative species.

Decisions as to which of these putative new species should be described *de novo* are based on the availability of a representative set of specimens for a single MOTU and congruence between the various species delimitation algorithms. In this we opt for a conservative approach, describing only those species for which we had (1) at least 3 sequences (specimens) for *cox*3, (2) at least sequences for the three markers (*cox*3, *rbc*L and *psb*A), and (3) which resulted in consensual results between analyses (GMYC, bGMYC and ABGD) and genes. In other words, we opted for the least inclusive species delimitation. Based on this rationale we describe 10 species *de novo* (*L. abaculusa, L. abscondita*, *L. densa*, *L. dimorpha*, *L. gibbera*, *L. hederacea, L. monticola*, *L. petila*, *L. rosacea*, and *L. undulata*) (TABLE 2 and FIG. 4). Although there are strong indications that several of the remaining MOTUs could well represent new species as well, at present they are left undescribed, awaiting additional sampling.

Morphology and ecology

A combination of morphological and ecological traits allows a good differentiation of the New Caledonian species. Combinations of morphological, anatomical and ecological characters are graphically represented in FIG. 7.

The *Lobophora* complex provides an excellent example of the power of molecular-assisted alpha taxonomy (MAAT; Cianciola et al. 2010) in which species are delimited based on molecular data and subsequently the diagnostic value of morphological and ecological characteristics reassessed (see also Verbruggen et al. 2005; Leliaert et al. 2014). Even though the current sampling most likely fails dramatically in representing the global species diversity in the genus, several trends with regard to the evolutionary signal of morphological characters stand out. Lineage A composed of five MOTUs, including the newly described species *L. rosacea* is characterized by

352 a decumbent or fasciculate thallus. Species of lineage B, composed of two species *L. nigrescens*
353 s.l. and *L. australis,* is characterized by erect thalli and conspicuous basal holdfasts. Members of
354 the lineage C, including the newly described species *L. hederacea*, *L. undulata*, *L. monticola* and
355 *L. abaculusa*, are commonly associated with corals and present a predominantly conk-like form.
356 *L. hederacea* may also adopt a crustose form especially when found covering specific coral
357 genera (*e.g. Seriatopora caliendrum* Ehrengerg (1834) and *S. hystrix* Dana (1846)). The species
358 of lineage E, including the species *L. dimorpha* and *L. pachyventera,* adopt predominantly a
359 procumbent form. The species of the lineages D and F, including the species *L. crassa*, *L.*
360 *abscondita*, *L. gibbera*, *L. densa*, *L. asiatica* and *L. petila,* are characterized by a predominant
361 crustose form. *L. papenfussii* from Bikini Atoll (Marshall Islands) and *L. rickeri,* from Lord
362 Howe Island (Australia), which presents a crustose form and a thick thallus, may well belong to
363 the lineage F, whose members share the same morphological characteristics (*i.e.* a crustose form
364 and thick thallus).
365 *Evolutionary perspective and ecological significance of the morphology*
366 The genus *Lobophora* illustrates the misapprehension of morphological differences for
367 phenotypic plasticity instead of genetic diversity well. Several authors (*e.g.* De Ruyter van
368 Steveninck et al. 1988; Littler and Littler 2000) already observed different growth forms and
369 certainly sensed the existence of different species in relation to the different forms, but nobody
370 ventured to look into this diversity until recently (Sun et al. 2012). The morphological diversity
371 observed within the genus *Lobophora* was until now considered as the phenotypic plasticity (*e.g.*
372 Coen and Tanner 1989, De Ruyter van Steveninck et al. 1988; Littler and Littler 2000) displayed
373 by a single species, namely *Lobophora variegata*. Today, three arguments strongly stand against
374 this misconception. First, recent studies including the present one unraveled the hotchpotch of

species hidden behind the catch-all species *Lobophora variegata*. Second, comparison of phylogeny and morphological results revealed the existence of predominant growth forms in each major lineage. Lastly, in a same habitat we may find different species with different forms. However, one cannot discard phenotypic plasticity off the picture, as we can observe a certain degree of plasticity in every species, with a spectrum of shapes ranging from crustose to erect, but yet again with a predominant form per species. By comparing the morphologies shared by species of a same lineage, we were able to distinguish predominant forms in each lineage. The most basal lineages (A and B) possess predominantly an erect form, the most recent lineages (D-F) present a procumbent to a crustose form, and the intermediate lineage (C) presents a decumbent form. Most likely the ancestral form was a *Zonaria*-like erect species with a single holdfast, which was also suggested in Sun et al. (2012). Furthermore, those forms seem to be associated with ecological features. In fact, *Lobophora* species are found to have a wide variety of habitat and substratum preferences in New Caledonia (*e.g.* bedrocks, coral rubbles, dead corals and live corals). More remarkably we noticed that this variety of substrata reflected a niche partitioning between the major lineages. For instance, species of lineage B are mostly found growing on sand bottoms, species of lineage C are strongly found in interactions with live corals. These species, present the capacity to bleach and overgrow corals, certainly by the means of secondary metabolites. Species of lineage A are also found in interactions with corals. Species of lineages D to F are mostly found on bedrocks, dead corals or coral rubbles.

Conclusion

The high levels of *Lobophora* diversity unveiled from a single locality in the Pacific Ocean raises important question with respect to the global diversity of the genus, the distributions and

range sizes of the individual species, as well as the mechanisms facilitating co-existence. Current sampling of *Lobophora* species does not allow to draw far ranging conclusions, but it would appear that individual *Lobophora* species are restricted to one ocean basin and in this aspect it reminisces the biogeography of the genus *Padina*, for which there is no or very scanty evidence for species spanning more than one ocean basin. Our analyses included two specimens from the Caribbean Sea, the type locality of *L. variegata*. Even though the presence of genuine *L. variegata* in the Indo-Pacific Ocean seems quite unlikely, additional sampling of the Caribbean region is highly needed to precisely determine the identity of *L. variegata* and assess the species diversity in the Atlantic Ocean. In addition, at present more than half of the MOTUs are recorded only from New Caledonia, but it remains unclear which percentage of the unveiled diversity is really restricted to the study area. An extensive sampling in the Indo-Pacific region is needed to improve our understanding of *Lobophora* distribution patterns significantly.

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598

599 **Tables**

600 Table 1. Comparison of species delimitation analyses.

601 Table 2. Descriptions of new *Lobophora* species from New Caledonian.

602

603 **Figures**

604 Figure 1. Map showing the sampling sites of *Lobophora* specimens around New Caledonia with
605 indication of the sampling effort and number of species collected per site.

606

607 Figure 2. Schematic representation of the various growth forms discerned in *Lobophora,* with the
608 circle representing the substratum. The center of the picture depicts the various *Lobophora*
609 growth forms on live or dead coral.

610

611 Figure 3. Schematic representation of a longitudinal and a transverse section of *Lobophora*,
612 illustrating the anatomical characters.

613

614 Figure 4. Results of the three species delimitation methods based on the *cox*3 dataset. Species
615 delimitation results of ABGD (inner), GMYC (middle) and bGMYC (outer) are represented by 3
616 concentric circles. The tree is the maximum clade credibility tree obtained from BEAST. Red
617 lines and asterisks indicate conflicting results between ABGD, GMYC-based methods and both
618 GMYC-based methods, respectively.

619

620 Figure 5. Boxplots of anatomical variables of New Caledonian *Lobophora* species; rectangles
621 and whiskers bound 25-75 percentiles and the 10-90 percentiles respectively, horizontal lines
622 show the median, circles are extreme values, red and blue points show the mean and standard
623 deviation respectively.

624

625 Figure 6. *Lobophora* species tree with indication of morphological and ecological characteristics
626 as well as the distribution of the MOTUs as presently known. Species represent the MOTUs
627 resulting from the species delimitation analyses. The tree is the maximum clade credibility tree
628 obtained from a BEAST analysis of the concatenated alignment of four genes (*rbc*L, *cox*3, *psb*A
629 and LSU). The values shown at each node represent Bayesian posterior probabilities (left part of
630 the circle) and ML boostrap values (right part of the circle) respectively. High support (posterior
631 probabilities > 0.95 and bootstrap values > 0.9) is indicated in black, while low support
632 (posterior probabilities < 0.95 and bootstrap values < 0.9) is indicated in gray. No color indicates
633 configuration incongruence between the Bayesian and the Maximum Likelihood trees.
634 Ecological codes: br: bedrock; cb: coral base; cc: crustose coralline algae; dc: dead coral; lc: live
635 coral; oa: with other algae; uc: unhealthy coral.
636
637 Figure 7. Schematic representation of the ecological (substrate preferences), morphological
638 (growth forms) and anatomical (log-transformed thallus thickness) features of the New
639 Caledonian *Lobophora* species. Horizontal dashed lines separate the substrates. * *L. nigrescens*
640 s.l. grows on hard substrates (*e.g.* rocks, bedrock) found in sandy bottoms.
641
642 Figure 8a-l. External morphology of New Caledonian *Lobophora* species. For new species the
643 picture represents the holotype. a. *L. gibbera* ; b. *L.* crassa; c. *L. densa* ; d. *L. abscondita* ; e. *L.*
644 *abaculusa*; f. *L. monticola*; g. *L. undulata*; h. *L. hederacea*; i. *L. rosacea*; j. *L. rosacea*. k. *L.*
645 *dimorpha*; l. *L. dimorpha;* m. *L. pachyventera*; n. *L. petila*; o. *L. nigrescens* s.l..
646

Figure 9a-f. Longitudinal (on the left) and transverse (on the right) sections of New Caledonian *Lobophora* species. a. *L.* gibbera; b. *L. crassa*; c. *L. densa*; d. *L. abscondita*; e. *L. abaculusa*; f. *L. monticola*; g. *L. undulata*;

Figure 10a-f. Longitudinal and transverse sections of New Caledonian *Lobophora* species (continued). a. *L. hederacea*; b. *L. rosacea*; c. *L. dimorpha*; d. *L. pachyventera*.; e. *L. petila*; ; f. *L. nigrescens* s.l..

Supplementary figures

Figure S1. Boxplots representing the log-transformed thickness values of New Caledonian *Lobophora* species. One Way ANOVA; Df = 20, F value = 788 and $p < 2.2e\text{-}16$. A Tukey HSD post-hoc test revealed significance groups, represented by letters. Rectangles and whiskers bound 25-75 percentiles and the 10-90 percentiles respectively, horizontal lines show the median, circles are extreme values, red and blue points show the mean and standard deviation respectively.

Figure S2. Maximum likelihood tree, generated with RAxML, based on the concatenation of *rbc*L, *cox*3, *psb*A and LSU sequences. The values shown at each node represent ML boostrap values.

Figure S3. Bayesian tree, generated with MrBayes, based on the concatenation of *rbc*L, *cox*3, *psb*A and LSU sequences. The values shown at each node represent the posterior probability values.

Supplementary tables

Table S1. Origin of the specimens used in this study and their GenBank accession numbers.

Table S2. List of primers used in this study.

Table S3. Comparison of species delimitation results for the three methods (GMYC, BI GMYC and ABGD) between the three genes (*cox*3, *rbc*L, *psb*A) on the specimen (left table) and species levels (right table). Asterisks indicate that the specimens separated by missing sequences are part of the same delimited species.

Table S4. Results of the ANOVA of nine anatomical traits for the New Caledonian *Lobophora* species.

Table S5. Results of the Tukey HSD post-hoc test.

Table S6. Comparison of morphological characters among species of *Lobophora*.