MEDITERRANEAN *LITHOPHYLLUM STICTIFORME* (CORALLINALES, RHODOPHYTA) IS A GENETICALLY DIVERSE SPECIES COMPLEX: IMPLICATIONS FOR SPECIES CIRCUMSCRIPTION, BIOGEOGRAPHY AND CONSERVATION OF CORALLIGENOUS HABITATS[1]

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Lithophyllum species in the Mediterranean Sea function as algal bioconstructors, contributing to the formation of biogenic habitats such as coralligenous concretions. In such habitats, thalli of Lithophyllum, consisting of crusts or lamellae with entire or lobed margins, have been variously referred to as either one species, L. stictiforme, or two species, L. stictiforme and L. cabiochiae, in the recent literature. We investigated species diversity and phylogenetic relationships in these algae by sequencing three markers (psbA and rbcL genes, cox2,3 spacer), in conjunction with

methods for algorithmic delimitation of species (ABGD and GMYC). Mediterranean subtidal *Lithophyllum* belong to a well-supported lineage, hereby called the *L. stictiforme* complex, which also includes two species described from the Atlantic, *L. lobatum* and *L. searlesii*. Our results indicate that the *L. stictiforme* complex consists of at least 13 species. Among the Mediterranean species, some are widely distributed and span most of the western and central Mediterranean, whereas others appear to be restricted to specific localities. These patterns are interpreted as possibly resulting from allopatric speciation events that took place during the Messinian Salinity Crisis and subsequent glacial periods. A partial *rbc*L sequence from the lectotype of *L. stictiforme* unambiguously indicates that this name applies to the most common subtidal *Lithophyllum* in the central Mediterranean. We agree with recent treatments that considered *L. cabiochiae* and *L. stictiforme* conspecific. The diversity of *Lithophyllum* in Mediterranean coralligenous habitats has been substantially underestimated, and future work on these and other Mediterranean corallines should use identifications based on DNA sequences.

Key index words: biogenic habitats; biogeography; coralline algae; *cox*2,3; cryptic species; ecosystem engineers; molecular phylogeny; *psb*A; *rbc*L

Abbreviations: BI, Bayesian inference; BP, Bootstrap value; *cox*2,3, spacer of the cytochrome oxidase subunit 2 (*cox*2) and 3 (*cox*3); GTR, general time reversible; ML, Maximum Likelihood; MSC, Messinian Salinity Crisis

In the last 10 years, there has been a renewed interest in the taxonomy and systematics of the coralline algae (Orders Corallinales, Hapalidiales and Sporolithales) fueled by the availability of DNA sequence data (Nelson et al. 2015, Caragnano et al. 2018). These data have become an essential tool in coralline studies and have led to major insights in the diversity and evolution of this group. Molecular phylogenetic and taxonomic investigations have drawn a new scenario in which some key points have emerged: (i) the diversity of coralline algae revealed by DNA sequence data is much higher than indicated by morpho-anatomical data, both in non-geniculate (Bittner et al. 2011, Kato et al. 2011, 2013, Mateo-Cid et al. 2014, Pardo et al. 2014, Adey et al. 2015, Basso et al. 2015, Hernandez-Kantun et al. 2015a, Hind et al. 2016, Gabrielson et al. 2018) and geniculate (Hind and Saunders 2013, Hind et al. 2014a, Pardo et al. 2015, Williamson et al. 2015) species; (ii) a substantial amount of cryptic diversity exists that has led to gross underestimation of species numbers in individual geographical regions (e.g., Hernandez-Kantun et al. 2016 estimated a species diversity likely two to four times greater for the genus *Lithophyllum* in each geographic region); (iii) many morpho-anatomical

features traditionally used to identify coralline species are not sufficiently reliable for identification purposes (Gabrielson et al. 2011, Kato et al. 2011, Hind and Saunders 2013, Hind et al. 2014a,b, 2016, Pardo et al. 2014, Adey et al. 2015); and (iv) great care should be used in the application of species names; in particular, the practice of identifying specimens without molecular data from a particular region with names of species described from a geographically distant region should be abandoned.

Over the last decade, taxonomic investigations of coralline algae incorporating DNA sequence data have been conducted in many regions, contributing substantially to the knowledge of their marine biodiversity (e.g., New Zealand, Broom et al. 2008; Tropical Pacific Ocean, Bittner et al. 2011, Kato et al. 2011; Northeast Pacific Ocean, Gabrielson et al. 2011, Hind et al. 2014a,b, 2016, 2018; Indo-Pacific Ocean, Caragnano et al. 2018, Gabrielson et al. 2018; western Indian Ocean, Basso et al. 2015; Subarctic and Boreal Atlantic, Adey et al. 2015; Northeast Atlantic Ocean, Pardo et al. 2014, 2017, Hernandez-Kantun et al. 2015a,b; Atlantic Iberian Peninsula, Pardo et al. 2015; Warm Temperate to Tropical Western Atlantic Ocean, Mateo-Cid et al. 2014, Sissini et al. 2014, Bahia et al. 2015, Richards et al. 2018; Gulf of Mexico, Richards et al. 2014, 2016; Caribbean Sea, Hernandez-Kantun et al. 2016). The Mediterranean Sea represents a remarkable exception. In this area, coralline algae have received much attention, but DNA sequence data are still fragmentary and insufficient to define species diversity and distributions in detail. *Hydrolithon rupestre* (Wolf et al. 2015) and encrusting specimens of *Phymatolithon calcareum* (Wolf et al. 2016) were recorded from the Mediterranean based on a combination of morpho-anatomical and DNA sequence data. Similarly, *Pneophyllum cetinaensis* was described from Croatia using DNA sequence and morpho-anatomical data (Zuljevic et al. 2016), and the encrusting intertidal species *Lithophyllum byssoides* was the subject of a recent phylogeographic study (Pezzolesi et al. 2017). All other DNA sequences of Mediterranean taxa were produced in studies with a wider scope, not specifically focused on the Mediterranean Sea (Walker et al. 2009, Bittner et al. 2011, Hernandez-Kantun et al. 2015a, 2016, Pardo et al. 2015, Peña et al. 2015, 2018, Rösler et al. 2016).

A detailed knowledge of the identity and evolutionary relationships of Mediterranean corallines is needed for some important reasons. The Mediterranean Sea is a major biodiversity hotspot (Coll et al. 2010, Pascual et al. 2017). This basin's geological and hydrological diversity, including a complex history starting from its Mesozoic Tethyan origin, has favored the establishment of substantial biological diversity, with ~17,000 recorded marine species (Coll et al. 2010). A remarkable genetic diversity has been documented for numerous animal species (Patarnello et al. 2007) and this is potentially also

the case for the comparatively understudied macroalgae. For corallines, it can be expected that the species diversity in the Mediterranean Sea will be much higher than suggested by traditional morpho-anatomy, a prediction that is partially supported by the results of some recent studies (Pardo et al. 2015, Peña et al. 2015, Pezzolesi et al. 2017). The role of the Mediterranean as a key area for the evolution of corallines has been highlighted by the recent discovery of the first freshwater species, *Pneophyllum cetinaensis*, from the river Cetina, Croatia (Zuljevic et al. 2016). Furthermore, in the Mediterranean some species of corallines are bioconstructors that play an exceptionally important role as ecosystem engineers (Ballesteros 2006, Pezzolesi et al. 2017, Ingrosso et al. 2018). Several bioconstructions produced by corallines have been described for the Mediterranean (Bressan et al. 2009, Ingrosso et al. 2018), among which coralligenous concretions are the best known and most studied. These habitats occur on subtidal Mediterranean rocky shores, where their overall extent is estimated to exceed 2,700 km^2 in surface area (Martin et al. 2014). They are built by accumulation of cemented encrusting thalli of *Lithophyllum, Lithothamnion, Mesophyllum, Neogoniolithon* and *Spongites* (Ballesteros 2006, Bressan et al. 2009). The ecological, economic, and recreational importance of coralligenous habitats has been extensively evaluated and acknowledged (Doxa et al. 2016, Tribot et al. 2016, Chimienti et al. 2017) and the numerous anthropogenic stressors that threaten them have been characterized in detail (Piazzi et al. 2012).

Lithophyllum is one of the most speciose genera of non-geniculate corallines and is represented in the Mediterranean by 16 currently accepted species (Cormaci et al. 2017). The generitype species, *L. incrustans*, was originally described from Sicily (Philippi 1837: 388). Species of *Lithophyllum* are the most important algal bioconstructors in the Mediterranean, in particular as the main contributors to coralligenous concretions (Garrabou and Ballesteros 2000, Ballesteros 2006, Rodriguez-Prieto 2016). Although these algae were already known to early naturalists (e.g., Zanardini 1843, Agardh 1852, Dufour 1861) and have been well-studied, their phylogenetic diversity and species circumscriptions remain poorly understood.

Specimens of *Lithophyllum* typical of the coralligenous and other subtidal habitats form flat or superimposed crusts not strongly adherent to the substratum. For a long time, they were referred to as a single species, *L. expansum* (or its homotypic synonym *Pseudolithophyllum expansum*), a species no longer classified in *Lithophyllum* as currently circumscribed (see Woelkerling 1983). The nomenclatural history of these corallines is long and difficult to disentangle, and several specific and intraspecific names have been used for them (Zanardini 1843, Agardh 1852, Dufour 1861, Hauck 1877, 1885, Foslie 1897,

De Toni 1905, Hamel and Lemoine 1953, Boudouresque and Verlaque 1978, Furnari et al. 1996, Athanasiadis 1999). However, the classification adopted in recent years is straightforward: most authors recognize only two species, *Lithophyllum stictiforme* (previously as "*stictaeforme*") and *L. cabiochiae* (previously as "*cabiochiae*") as taxonomically valid (Athanasiadis 1999, Rodriguez-Prieto et al. 2013, Guiry and Guiry 2018). Names used in the past, particularly by early authors, are now considered synonyms of *L. stictiforme*. Some treatments adopt an even simpler scheme, considering *L. stictiforme* and *L. cabiochiae* conspecific, with *L. stictiforme* having nomenclatural priority (Cormaci et al. 2017:261-262).

To date, all taxonomic studies on Mediterranean *Lithophyllum* from coralligenous or other deep subtidal habitats have been based on morpho-anatomical data. Only seven sequences are available in GenBank, deposited as "Uncultured Corallinales" by Bittner et al. (2011) or as *L. stictaeforme* by Hernandez-Kantun et al. (2016) and by Rösler et al. (2016). These data represent a useful starting point, but they are insufficient for a detailed phylogenetic and taxonomic assessment. We present here a large-scale molecular phylogenetic assessment of this group of coralline algae, based on samples obtained from a wide geographical range in the western and central Mediterranean.

MATERIALS AND METHODS

Field surveys and sample collection. Samples of *Lithophyllum* were collected from 27 localities in the western and central Mediterranean (Table S1 in the Supporting Information; Fig. 1). Samples targeted for this study were subtidal specimens with a gross morphology corresponding to material identified in the last decades as *Lithophyllum expansum* (=*P. expansum*), *L. frondosum, L. cabiochiae* or *L. stictiforme*. Specimens formed crusts, blades or lamellae of variable thickness, usually not strongly adherent to the substratum, with entire or lobed margins and smooth surfaces (often formed by several superimposed lamellae in large specimens; Fig. 2). All samples were removed from rock using a sharp knife or hammer and chisel (except the samples LLG4596D and NAMESSINA, that consisted of small specimens growing on fragments of discarded fishing lines; Fig. 2M). Samples were collected by snorkeling or SCUBA diving, at depths between 0.2 and 60 m (mainly between 15 and 40 m). Entire or large fragments of specimens were air-dried and then placed in ziploc bags with silica gel. Voucher specimens were deposited in TSB (section of calcareous algae), PC, and SANT (Table S1, herbarium abbreviations follow Thiers 2018). In addition, some historical herbarium specimens relevant to the study were obtained and used for DNA extraction. A fragment of the lectotype of *Melobesia stictiformis* was obtained from S by PWG and processed by JRH. Fragments of the lectotype of *Melobesia frondosa*, the lectotype of *Melobesia grandiuscula*, and a syntype of *Lithophyllum lobatum* (Børgesen 3277 - PC), all deposited in PC, were processed by LLG and VP.

Histological sections and SEM. Morpho-anatomical characters were studied by examination of decalcified histological sections and SEM observations. Serial histological sections were prepared following Basso and Rodondi (2006). SEM methods followed Kaleb et al. (2018) and Peña et al. (2018).
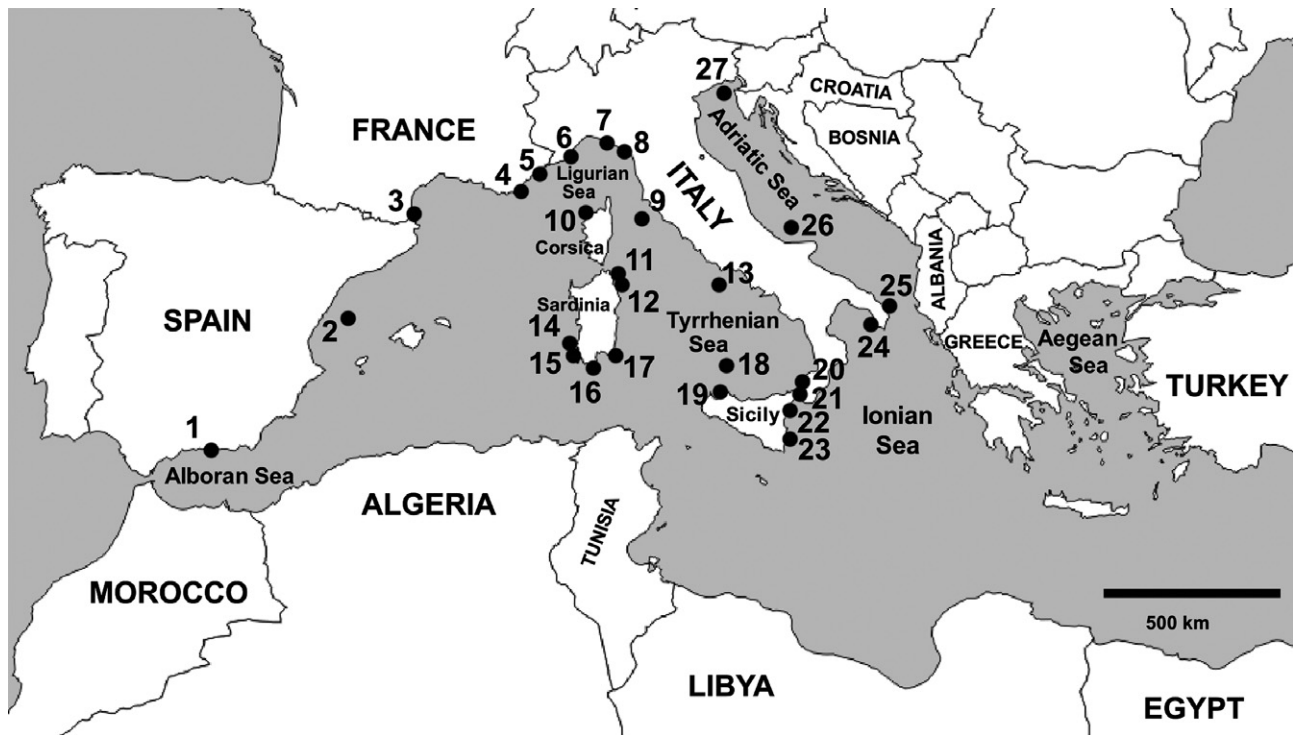
Fig. 1. Map of Mediterranean Sea showing sampled localities (numbers). 1 La Herradura; 2 Islas Columbretes; 3 Banyuls-sur Mer; 4 Port-Cros; 5 Rade de Villefranche; 6 Gallinara Island; 7 Portofino; 8 Bonassola; 9 Giannutri Island; 10 Calvi; 11 Tavolara Island; 12 Molara Island; 13 Ponza Island; 14 Buggerru; 15 Masua; 16 Capo Spartivento; 17 Cala Regina; 18 Ustica Island; 19 Castellammare del Golfo; 20 Scilla; 21 Torre Faro; 22 Acireale; 23 Ognina Island; 24 Santa Caterina; 25 Otranto; 26 Tremiti Islands; 27 Trieste.

DNA extraction, amplification, and sequencing. For field-collected specimens, DNA extractions were performed on silica-dried material. When necessary, the material was carefully cleaned (with a toothbrush, a scalpel or forceps) to remove epiphytes growing on the upper surface and invertebrates growing underneath or in small cavities. For samples processed at the Università Politecnica delle Marche (UNIVPM samples, deposited in TSB; see Table S1), DNA was extracted following the modified protocol of the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Crawley, UK) by Broom et al. (2008). For field-collected samples processed at the Museum National d'Histoire Naturelle (MNHN samples, deposited in PC and SANT; see Table S1), DNA was extracted using the NucleoSpin® 96 Tissue kit (Macherey-Nagel, GmbH and Co. KG, Germany) following the manufacturer's protocol. The PC type collections (*Lithophyllum lobatum*, *Melobesia frondosa*, and *M. grandiuscula*) were extracted using the QIAampDNA Micro Kit (Qiagen S.A.S., Les Ulis, France) following the manufacturer's protocol for tissues. For UNIVPM samples, the *psb*A gene and the *cox*2,3 spacer were PCR-amplified using the methods of Pezzolesi et al. (2017). PCR products were visualized and quantified in 1.5% agarose gels stained with Gel-RedTM (Biotium, Hayward, CA, USA) using Low DNA Mass Ladder (Invitrogen, Carlsbad, CA, USA) as reference, and visualized under UV light. PCR products with expected lengths and yields were purified and sequenced by Macrogen Europe (Amsterdam, The Netherlands). For MNHN samples, the *psb*A gene and the *cox*2,3 spacer were amplified following Peña et al. (2015) and Zuccarello et al. (1999) respectively. PCR products were purified and sequenced by Genoscope (Bibliothèque du Vivant program, Centre National de Séquençage, France) and by Eurofins (Eurofins Scientific, France). PCR amplification and sequencing of the *rbc*L gene were performed at the University of North Carolina, Chapel

Hill, by PWG on selected UNIVPM samples (for which the DNA was provided by FR), following the methods of Gabrielson et al. (2011).This marker was also amplified and sequenced for some selected MNHN samples by LLG following the protocol of Gabrielson et al. (2011). The lectotype fragment of *Melobesia stictiformis* was extracted, PCR-amplified and sequenced, and the sequences were edited by JRH following the protocols of Hernandez-Kantun et al. (2016) and Hughey and Gabrielson (2012), except that the reverse amplification and sequencing used the reverse primer 1308Cor: 5′-GTCCTTCATTTCTTGC TAAAACC-3′.

Sequence alignments and phylogenetic analyses. The quality of the sequences was assessed by visual examination of the electopherograms in Sequence Scanner 1.0 (Applied Biosystems, Waltham, MA, USA) or CodonCode Aligner® (Codon-Code Corporation, Centerville, MA, USA). Only high-quality sequences (i.e., devoid of double peaks or other ambiguities that could make the readings dubious) were included in the alignments for phylogenetic analyses. The alignments used in the study were assembled after a series of preliminary phylogenetic analyses aimed at a correct selection of ingroup and outgroup taxa. These analyses indicated that the samples sequenced in this study belonged to a strongly supported clade recovered in the recent *psb*A phylogenies of Hernandez-Kantun et al. (2016) and Richards et al. (2018); this clade is hereby referred to as *Lithophyllum stictiforme* complex and the sequences belonging to it were considered the ingroup. The results of Hernandez-Kantun et al. (2016) and Richards et al. (2018) also indicated that the *L. stictiforme* complex is sister with strong support to a clade formed by North Atlantic *L. bathyporum*, *L. dentatum*, *L. hibernicum*, and *L. incrustans*. Therefore, *psb*A and *rbc*L sequences of these species (Hernandez-Kantun et al. 2015b) and *cox*2,3
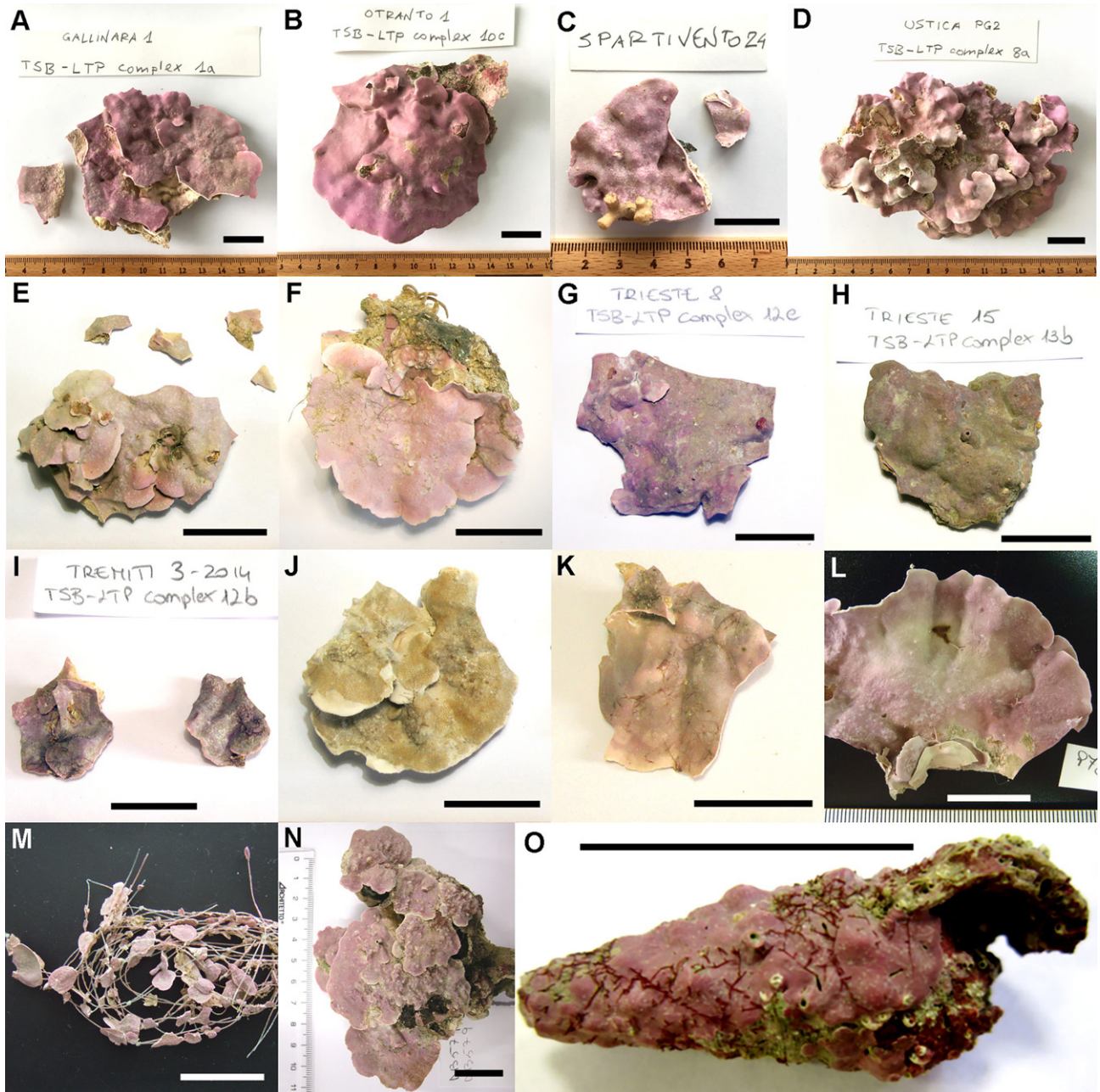
Fɪɢ. 2. Habit of selected sequenced samples of *Lithophyllum stictiforme* complex. (A). Gallinara1 (clade 1). (B) Otranto1 (clade 1). (C) Spartivento24 (clade 1). (D) Ustica PG2 (clade1). (E) Giannutri2 (clade2). (F) Ponza22 (clade2). (G) Trieste8 (clade 3). (H) Trieste15 (clade 3). (I) Tremiti3-2014 (clade 4). (J) Giannutri3 (clade 5). (K) Tremiti6 (clade 6). (L) VPF00196 (clade 7). (M) NAMESSINA (clade 8). (N) DB579 (clade 9). (O) Holotype of *Lithophyllum searlesii* (clade 13). Scale bar for all images: 2 cm.[Color figure can be viewed at wileyonlinelibrary.com]

sequences of *Lithophyllum* sp. (Hernandez-Kantun et al. 2014, subsequently identified as *L. hibernicum*) were chosen as outgroup taxa in the respective data sets (see full list in Table S2 in the Supporting Information). Alignments were assembled separately for *psb*A, *rbc*L and *cox*2,3 using ClustalW in SeaView version 4 (Gouy et al. 2010) with default settings and refined by eye. In addition, a concatenated alignment *psb*A-*cox*2,3 was prepared for 62 samples for which both markers were sequenced, and a concatenate alignment *psb*A-*rbc*L for 25 samples. Neighbor-Joining (NJ) distance analyses were performed on all data sets using uncorrected

p-distances in SeaView version 4 (Gouy et al. 2010), with nodal support assessed by 1,000 bootstrap (BP) resamplings. Phylogenetic inference by Maximum Likelihood (ML) was performed using PhyML in SeaView version 4 (Gouy et al. 2010) and RAxML1.3 (Mac version, Silvestro and Michalak 2012). For the RAxML analyses, the *psb*A and *rbc*L data sets were partitioned by codon position (1st codon position +2nd codon position + 3rd codon position).The concatenated *psb*A-*cox*2,3 data set was partitioned in four partitions (*cox*2,3+ the three codon positions of *psb*A) and the concatenated *psb*A-*rbc*L data set in six partitions (the three codon

positions for both genes).The GTR+G model was used for all reconstructions (for each partition in the partitioned analyses, and for the whole data set in the single-locus *cox*2,3 analysis). Statistical support was inferred from 1,000 RAxML resamplings. Bayesian inference (BI) was applied for the three single-marker data sets and the two concatenated data sets in MrBayes v. 3.2.2 (Huelsenbeck and Ronquist 2001) using the model GTR+G+I for each of the partitions (as described above). Four Monte Carlo-Markov chains were run for 5 million generations, and trees were sampled every 1,000 generations. Tracer v.1.5 (Rambaut and Drummond 2007) was used to verify the stationary distribution of the runs; 1,250 trees were discarded as burn-in, using the remaining to build the 50% majority-rule consensus trees.

Analyses for algorithmic delimitation of species. Hypotheses for delimitation of species were built applying a General Mixed Yule Coalescent (GMYC) model (Fujisawa and Barraclough 2013) and the Automatic Barcode Gap Discovery (ABGD) tool (Puillandre et al. 2012; http://wwwabi.snv.jussieu.fr/public/abgd/) to the *psb*A and *rbc*L data sets. For GMYC, the ultrametric tree derived from Bayesian phylogenetic analyses of the *psb*A alignment run in BEAST v1.7.4 (Drummond et al. 2012) under the GTR+G+I model, an uncorrelated log normal (UCLN) relaxed molecular clock, and a coalescence tree prior, were used. In BEAST, two Monte Carlo Markov Chains (MCMC) analyses were run for 10 million generations, sampling every 1,000th generation. The information from a sample of trees was summarized onto a single "target" tree (10% burn-in discarded at the start of the run, 0.5 of posterior probability limit of the nodes in target tree) using Tree Annotator v 1.7.4 (http://beast.bio.ed.ac.uk). GMYC analyses were performed using the SPLITS package for R (http://r-forge.r-project.org/project/splits). Hypotheses of species delimitation with the ABGD tool were generated by evaluating the results of analyses performed under Jukes-Cantor (JC69), Kimura (K80) TS/TVor simple distances, with Pmin of 0.001, Pmax of 0.1, 10 steps, and relative gap widths of 1.0 (for *rbc*L) or 1.5 (for *psb*A).

RESULTS

Molecular phylogeny and species delimitations. This study produced 85 *psb*A, 26 *rbc*L and 75 *cox*2,3 new sequences respectively (Table S1). Among historical specimens, a partial *rbc*L sequence (118 bp) was obtained from the lectotype material of *Melobesia stictiformis* (basionym of *Lithophyllum stictiforme*) and a*psb*A sequence was obtained from a syntype specimen of *L. lobatum*. Unfortunately, no sequences could be generated for types of *M. frondosa* and *M. grandiuscula*.

Single-marker phylogenies were congruent in both topology and statistical support (Figs. 3–5). In these phylogenies, the *Lithophyllum stictiforme* complex was recovered as a strongly supported lineage that included all samples sequenced in this study. The complex was formed by numerous clades, which were generally well-supported in all phylogenetic trees inferred. The relationships in the basal nodes of the trees, however, were usually weakly supported.

The *psb*A alignment was 910 bp long. This was the marker for which the highest number of sequences was obtained (94 ingroup + 6 outgroup). GMYC analyses performed on the *psb*A data set did not provide significant results. The ABGD analyses performed on *psb*A separated the ingroup into a number of species ranging from 13 (Kimura K80, partition with prior maximal distance $P = 4.64\text{e-}03$; or distance simple, partition with prior maximal distance $P = 1.00\text{e-}03$) to 33 (Kimura K80, partition with prior maximal distance $P = 1.00\text{e-}03$). The 13 species detected in the most conservative estimate corresponded to 13 highly supported clades recovered by the phylogenetic analyses (Fig. 3).Three of these clades contained the majority of the samples and could be characterized in terms of morphology and distribution. Clade 1, which represents the genuine *Lithophyllum stictiforme* (based on *rbc*L results, see below) included 35 samples from the central Mediterranean area (French Riviera, Corsica, Sardinia, Sicily, and Ligurian, Tyrrhenian and Ionian shores of Italy).This clade included the largest-sized specimens collected in the study, with the habit of thick lobed lamellae (often superimposed) with a smooth to undulate surface, violet, magenta, or dark pink in color (Fig. 2, A–D). Samples were collected mostly between −20 and −50 m depth. Clade 2 comprised 17 samples from the western and central Mediterranean, with a geographic distribution largely overlapping that of clade 1 (extending to the central coast of Spain and not recorded in Ionian Italy, but otherwise identical).The depth range of collection was also similar (mainly between −15 and −50 m). Samples of this clade consisted of thin foliose lamellae, single, or superimposed, with a smooth or slightly corrugated surface, bright to light pink in color (Fig. 2, E and F). Clade 3 included two highly supported sister lineages with different geographic distributions, i.e., a subclade of seven samples from the Adriatic Sea (Trieste, Italy; 3A) and a subclade of seven samples from the western Mediterranean (France, Ligurian Sea and Sicily; 3B).Specimens of clade 3 were encrusting, adherent to the substratum, with a surface slightly corrugated, grayish violet to dark pink in color (Fig. 2, G and H); this clade included the thickest specimens recorded in the study (up to 2,500 μm). For the clades 1, 2, and 3, no variation in color related to depth was noted (even when the depth range was wide, as it was the case for clades 1 and 2).For the other 10 clades, morphology and distribution could not be characterized in detail due to the limited number of samples available. However, some features of the habit, ecology, and geographic distribution appeared potentially useful for the circumscription of some clades. For example, clade 8 differed markedly from all others in terms of habit (small, thin lamellae up to 1 cm wide) and substratum (discarded fishing lines, in contrast with the stable hard substrata occupied by all other samples; Fig. 2M). Clade 10 comprised specimens solely collected in the shallow subtidal zone (−0.5 m). Some clades appeared to have a restricted geographic distribution, i.e., clade 6 was recorded only
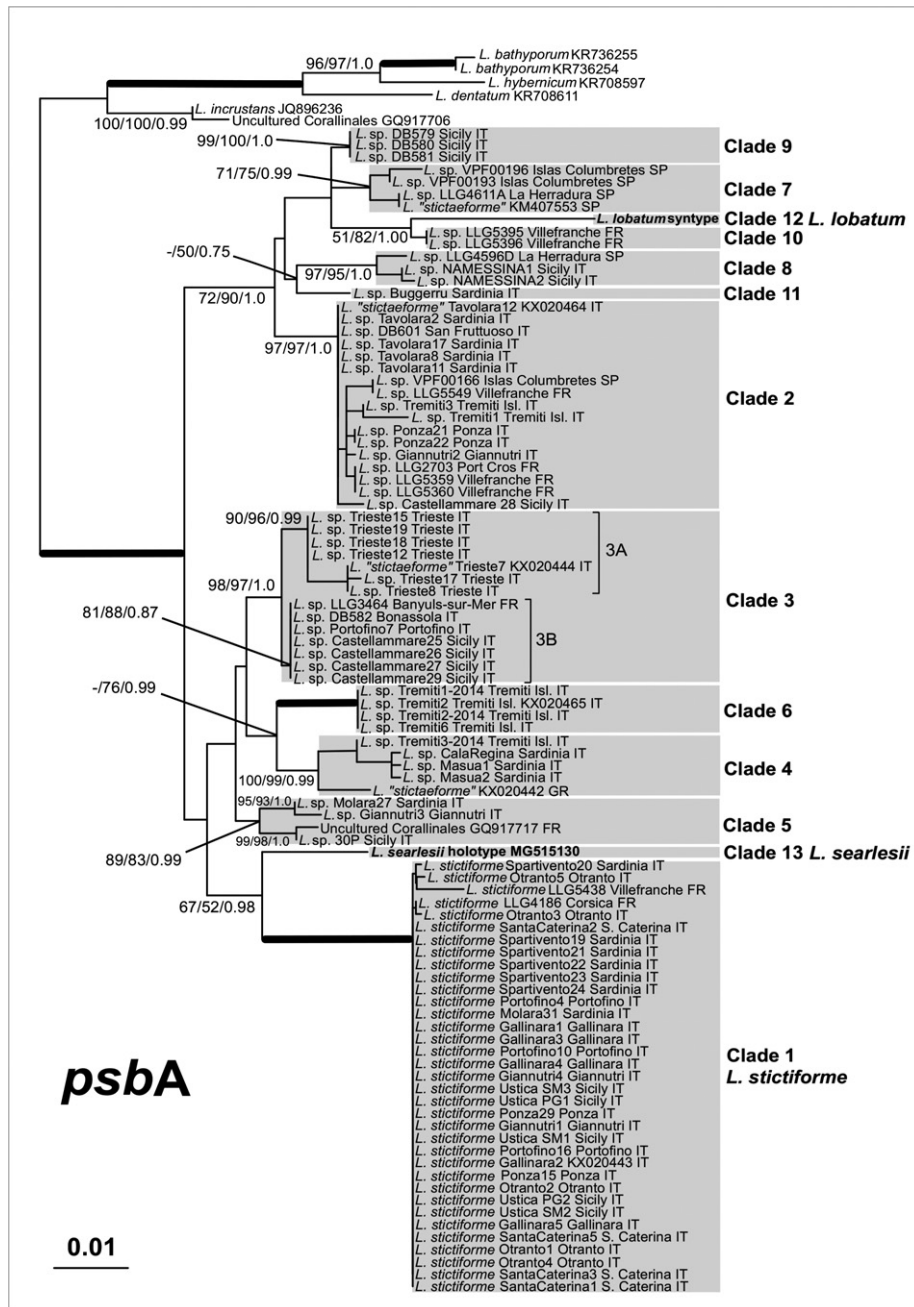
Fɪɢ. 3. Phylogram inferred from PhyML analysis of *psb*A data set for *Lithophyllum stictiforme* complex. Sequences obtained from type specimens marked in bold. Bootstrap support (BP) and Bayesian posterior probabilities (PP) indicated at nodes. Support values from left to right: BP for NJ, BP for RAxML, and Bayesian PP. BP values lower than 50% and PP lower than 0.8 not shown. Thick lines indicate full support (100% BP for both NJ and RAxML and 1.0 PP). Gray boxes indicate clades corresponding to 13 species detected by ABGD in the most conservative hypothesis. Scale number indicates substitutions/site.

from the Tremiti Islands (Adriatic Sea) and clade 7 only from central and southern Spain. Two clades (12 and 13) were represented by single sequences obtained from extra-Mediterranean material. Clade 12 corresponded to *L. lobatum*, for which the *psb*A sequence was generated from a syn type specimen in PC (from Puerto Orotava, Tenerife, Canary Islands). Clade 13 corresponded to *L. searlesii*, recently described from the western Atlantic

(Onslow Bay, North Carolina) and represented by the sequence of the holotype specimen (Richards et al. 2018; Fig. 2O).

Within the *psb*A ingroup, pairwise uncorrected distances were mostly in the range 2%–5%. The highest divergence was 5.72%, between the *Lithophyllum lobatum* syntype (clade 12) and *L. 'stictiforme'* KX020442 (from Voula Beach, Greece, clade 4).
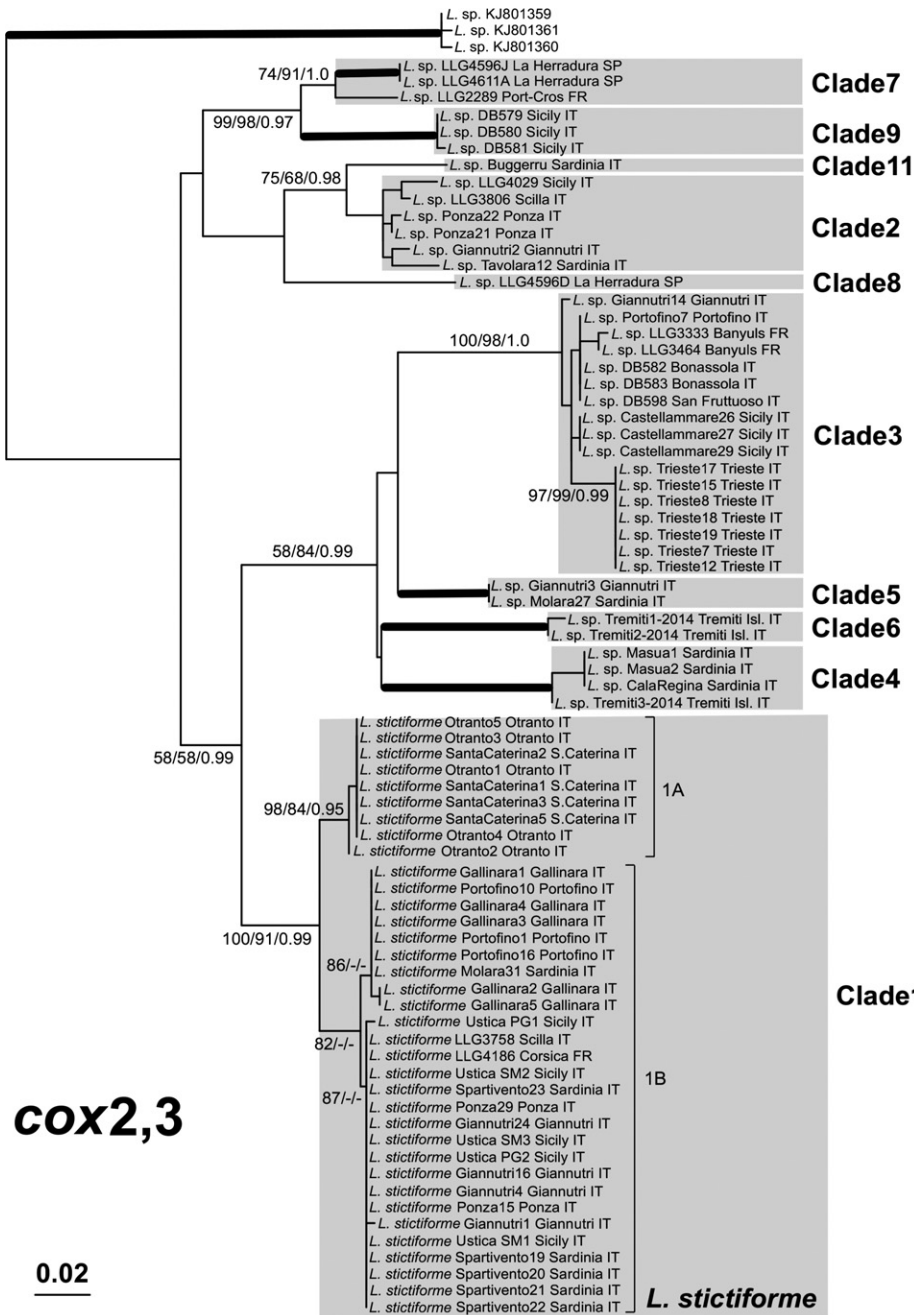
FIG. 4. Phylogram inferred from PhyML analysis of *cox*2,3 data set for *Lithophyllum stictiforme* complex. Bootstrap support (BP) and Bayesian posterior probabilities (PP) indicated at nodes. Support values from left to right: BP for NJ, BP for RAxML, and Bayesian PP. BP values lower than 50% and PP lower than 0.8 not shown. Thick lines indicate full support (100% BP for both NJ and RAxML and 1.0 PP). Gray boxes indicate clades corresponding to species recognized in *psb*A phylogeny. Scale number indicates substitutions/site.

The *cox*2,3 alignment was 372 bp long. The *cox*2,3 phylogeny (Fig. 4) did not include three clades represented in *psb*A (10, 12 and 13), because unfortunately *cox*2,3 sequences could not be generated for samples from these lineages. The absence of these lineages caused some minor differences in the topology of the trees. However, the composition of the clades agreed with *psb*A and the support values were similar. In the case of the *Lithophyllum stictiforme* clade

(clade 1, represented by 36 samples), the higher substitution rate of the *cox*2,3 provided results with better geographic resolution. Samples from the Ionian Sea (Otranto/Santa Caterina) formed a well-supported subclade (1A) separated from a subclade (1B) including the other samples (in which a further separation occurred between Ligurian and Tyrrhenian samples; this, however, was statistically supported only in distance analyses). Pairwise distances for the *cox*2,3 in
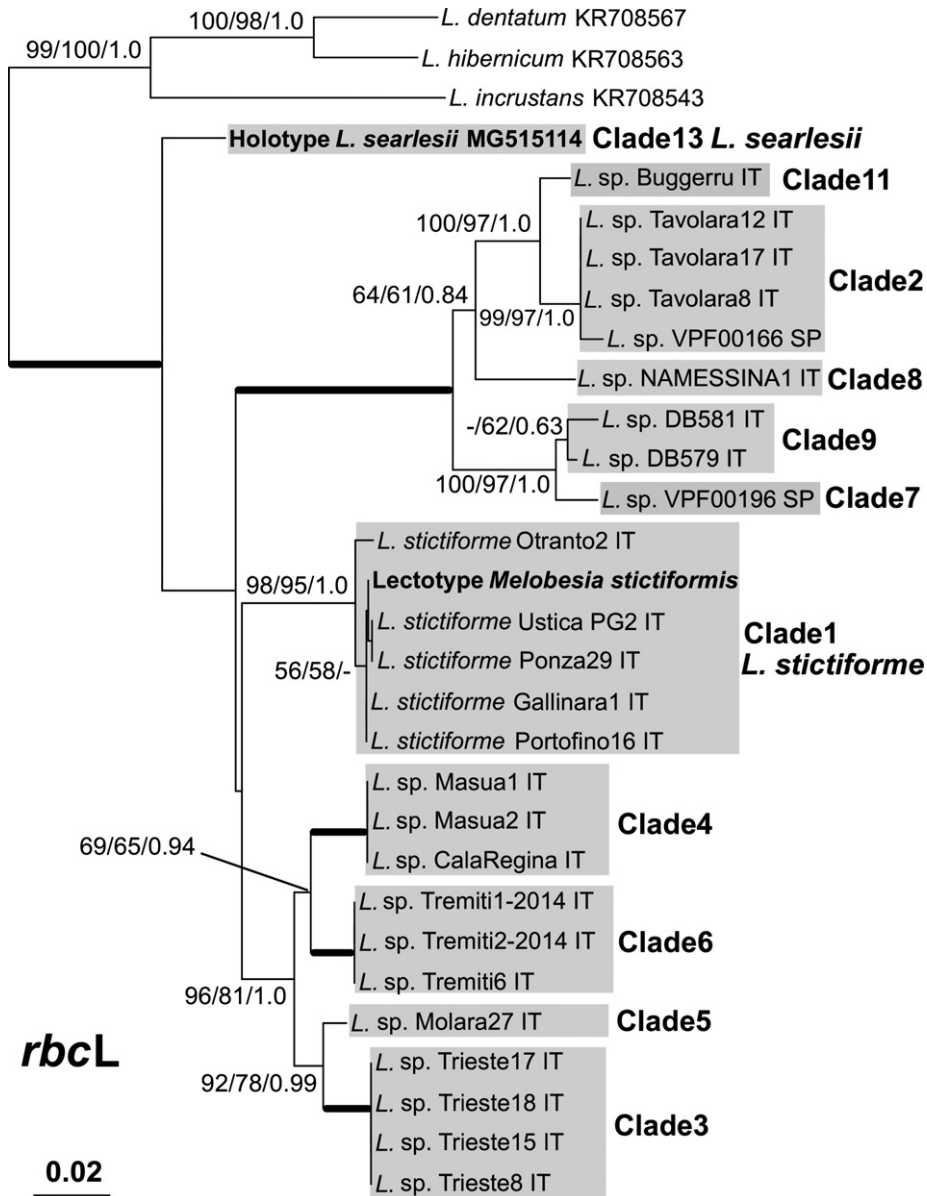
FIG. 5. Phylogram inferred from PhyML analysis of *rbc*L data set for *Lithophyllum stictiforme* complex. Sequences obtained from type specimens marked in bold. Bootstrap support (BP) and Bayesian posterior probabilities (PP) indicated at nodes. Support values from left to right: BP for NJ, BP for RAxML, and Bayesian PP. BP values lower than 50% and PP lower than 0.8 not shown. Thick lines indicate full support (100% BP for both NJ and RAxML and 1.0 PP). Shaded boxes indicate clades corresponding to species recognized in *psb*A phylogeny. Scale number indicates substitutions/site.

the *Lithophyllum stictiforme* complex ranged mostly between 6% and 11% with the greatest divergence (13.7%) between samples from the clade 4 (Masua1, Masua2, CalaRegina, Tremiti3-2014) and clade 7 (LLG4611A, LLG4596J).

The *rbc*L alignment was 690 bp long and provided equivalent topologies and similar support values (Fig. 5) as the other two markers. It was not possible to obtain the same phylogenetic coverage as *psb*A, because *rbc*L sequences could not be produced for clades 10 and 12. The GMYC analysis performed on the *rbc*L provided significant results and subdivided the *Lithophyllum stictiforme* complex into 15 species

(Fig. 6). ABGD analyses performed with different distance methods recovered the number of species ranging from 9 (simple distance, initial partition with prior maximal distance $P = 1.29e-02$; Fig. 6) to 14 (Kimura K80, partition with prior maximal distance $P = 1.00e-03$). Pairwise distances for *rbc*L sequences ranged mostly between 4% and 8%, with the greatest divergence (9.9%) between the samples from Trieste (clade 3) and the sample VPF00196 (clade 7).

A key finding from the *rbc*L sequences was the unambiguous assignment of the lectotype of *Melobesia stictiformis*, basionym of *Lithophyllum sticti-forme*, to clade 1 with strong support (Fig. 5). The
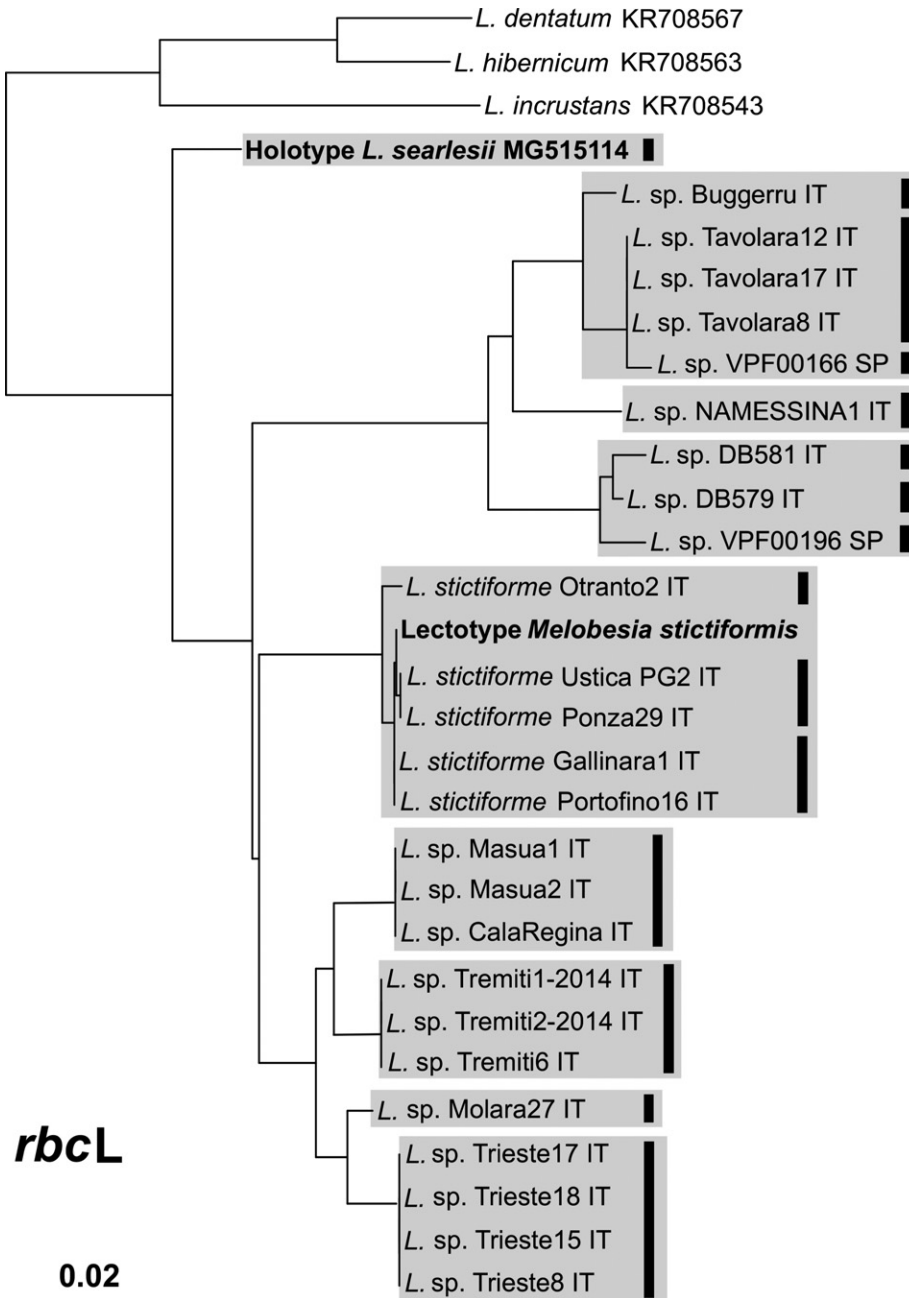
Fig. 6. Results of species delimitation analyses mapped on *rbc*L tree. Gray boxes indicate species detected by ABGD in the most conservative hypothesis. Black vertical lines indicate species detected by GMYC. Lectotype specimen of *Melobesia stictiformis* was excluded from species delimitation analyses due to limited characters available (118 bp), so a black vertical line does not appear beside this sample name. Scale number indicates substitutions/site.

118 bp *rbc*L sequence obtained from the lectotype of *Melobesia stictiformis* (Fig. S1 in the Supporting Information) was an exact match to specimens in clade 1 (Fig. 5). This sequence differed by 1 bp from the sample Otranto 2 and by 8 or more bp from specimens in all other clades, clearly indicating that clade 1 is the lineage to which the name *M. stictiformis* (and therefore *Lithophyllum stictiforme*) applies.

The analyses performed on the concatenated data sets inferred the same clades found in the single-marker analyses, with generally higher statistical support (Figs. S2 and S3 in the Supporting Information). In the case of the *psb*A-*rbc*L data set, concatenation also improved the resolution in the internal nodes of the trees, allowing discrimination of three well-supported main lineages: (i) *Lithophyllum stictiforme* clade, as recognized in single marker analyses; (ii) a superclade containing clades 2, 7, 8, 9, and 11; and (iii) a superclade containing clades 3, 4, 5, and 6 (Fig. S3). These lineages were

also recovered in the single marker analyses and in the concatenated *psb*A-*cox*2,3 analyses, but generally with lower support (Figs. 3–5, Fig. S2).

Gross morphology and anatomy. Details of gross morphological and morpho-anatomical characters for 12 of the 13 clades are summarized in the Table 1 and Table S3 in the Supporting Information, respectively (for clade 11, represented by a single sample from Buggerru, Sardinia, detailed observations were not possible due to the scant amount of material left after DNA extraction). In habit, the specimens examined in the study showed some variation in color, size, thickness, and shape of margin (Table 1; Fig. 2). Small thalli consisted of thin lamellae, discoid or fan-like in shape. Large thalli (particularly those of clade 1) were often formed by superimposed lamellae with several marginal and superficial lobes, with flat or undulate surface. In many specimens, there were concentric lines along the lower surface near the margin; this character, however, did not show a clear taxonomic pattern: it was observed in specimens belonging to many different clades and showed intra-clade variation (i.e., within a same clade it was evident in some specimens and absent or not evident in others). In some large thalli (mainly of clade 1), radial lines running from the center of the lower surface to the margin were also present; this character, however, was also not taxonomically informative. In many specimens (of several different clades) foliose lamellae or knobby excrescences protruded from the lower surface of the thalli.

All specimens conformed to the morpho-anatomical characters defining the genus *Lithophyllum* (as per Basso et al. 2014; Fig. 7). Thallus construction was dimerous, with crusts adhering to the substratum by a monostromatic hypothallus consisting of a layer of radially expanding filaments. In longitudinal radial section, the cells of the hypothallus were square, rhomboid or rectangular (Fig. 7A) and non-palisade, giving rise to ascending perithallial filaments from their dorsal surfaces (Fig. 7, A and B). Perithallial cells were square, rectangular or rhomboid, with adjacent filaments joined by secondary pit connections (Fig. 7C). In some thick sections, the thallus appeared monomerous, apparently as a result of several subsequent phases of secondary growth by perithallial filaments (Fig. 7, D and E). The dimerous organization was obscured at the margins of the thallus, where the perithallial filaments became parallel to the surface (Fig. 7E). Furthermore, in parts of the thallus that did not adhere to the substratum or were mechanically damaged, struts (i.e., groups of descending filaments) were frequently observed arising from the ventral surface (Fig. 7F); they produced the foliose lamellae or knobby growths observed on the lower surface. Trichocytes occurred in the perithallus of specimens from some clades (1, 5, 7); when present they were single, occasionally occurring one above another (Fig. 7G). The epithallus comprised 1–2 layers of flattened cells (Fig. 7H).

Reproductive specimens bore uniporate conceptacles with pores that were either flush with the surrounding thallus surface or just slightly protruding. Most reproductive thalli examined for morpho-anatomy were tetra/bisporangial. The tetra/bisporangial conceptacles were dumbbell-shaped (Fig. 7I) due to the remains of a more or less well-developed central columella. Pore canals were usually tapered from bottom to the top, but also cylindrical in some specimens of clades 1 and 2. Roofs of tetra/bisporangial conceptacles were typically 4–12 cell layers thick. Buried tetra/bisporangial conceptacles were observed in specimens of several clades (1, 2, 3, 5, 7), sometimes in large numbers (Fig. 7J). The few sporangia that were observed were peripherally arranged, mostly tetrasporangial, zonately divided and elliptical in shape (Fig. 7I). Mature gametangial conceptacles were rarely observed. Female gametangial conceptacles in side view were triangular (Fig. 7K), elliptical or dumbbell-shaped, with a roof formed by 5–10 cells; mature carpospores were very rarely observed, and were 20–25 μm in diameter. Male gametangial conceptacles were conical, with pore canals distinctly tapering from the bottom to the top and with roofs 3–4 cell layers thick; in mature conceptacles, spermatangia were restricted to the floor of the conceptacle chamber (Fig. 7L).

DISCUSSION

Species delimitation. The new data show that *Lithophyllum stictiforme* represents yet another example in which species diversity is substantially higher than suggested by morphology/morpho-anatomy alone, a situation that is now well-documented for marine macroalgae worldwide (Leliaert et al. 2009, Tronholm et al. 2012, Lee et al. 2013, Payo et al. 2013, Silberfeld et al. 2014, Vieira et al. 2014, 2017). In recent years, cryptic diversity has become a key topic in evolutionary biology and examples of this diversity have been demonstrated in numerous groups of organisms, including those that are complex and large-sized (Fišer et al. 2018).Theoretical considerations suggest that cryptic diversity can be expected to abound in marine macroalgae, particularly in forms with simple morphologies (Verbruggen 2014). This prediction has been confirmed in recent years, sometimes to extreme levels, especially when methods for algorithmic delimitation of species have been used (e.g., Payo et al. 2013, Silberfeld et al. 2014, Vieira et al. 2014, 2017).Corallines are no exception. Although their morpho-anatomical structure offers more characters for species discrimination compared to most other red seaweeds, they are perhaps the group of rhodophytes in which this situation is most pervasive. Numerous studies have revealed cryptic diversity at the species and/or genus ranks (Broom et al. 2008, Walker et al. 2009, Kato et al.

TABLE 1. Summary of external morphological characters and vegetative anatomical characters for the clades recovered in the molecular phylogenies of the *Lithophyllum stictiforme* complex. Clade 11 consisted of a single sample (*Lithophyllum* sp. Buggerru) for which detailed morpho-anatomical observations were not possible due to the scant amount of material available. For trichocytes: _ = absent; + = abundantly present; (+) = occasionally present.

	Clade 1 - *L. stictiforme*	Clade 2	Clade 3	Clade 4	Clade 5	Clade 6	Clade 7	Clade 8	Clade 9	Clade 10	Clade 12 - *L. lobatum*[a]	Clade 13 - *L. searlesii*[a]
Habit	Thick foliose lamellae, single or superimposed, with smooth or undulate surface	Thin foliose lamellae, single or superimposed, with smooth or slightly corrugated surface	Thick crusts with surface slightly corrugated	Thin superimposed lamellae with undulate surface	Thin lamellae with smooth or slightly corrugated surface	Thin, smooth lamellae	Thin foliose superimposed lamellae	Thin, foliose, smooth, flat lamellae	Thin, foliose superimposed lamellae	Encrusting, superimposed lamellae	Lobed crusts with knobby surface	Thin crusts, smooth to lumpy surface
Size (length and width)	Up to 25 cm	Up to 6–7 cm	Up to 10 cm	Up to 7–8 cm	Up to 7–8 cm	Up to 6–7 cm	Up to 8 cm	3–4 cm	Up to 15 cm	4–5 cm	Up to 3 cm	Up to 6–7 cm
Color of living thalli	Violet, magenta, dark pink	Light to bright pink	Grayish violet to dark pink	Grayish violet to mauve	Dark to grayish pink	Grayish violet to mauve	Grayish pink	Pink	Dark to grayish pink	Pink, pink mauve	Grey	Dark red
Concentric lines on lower surface	Usually present	Usually present	Evident in some samples, absent in others	Usually present	Present	Present	Present	Absent		Present	Not reported	Not reported
Radial lines on lower surface	Evident in some samples, absent in others	Absent	Absent	Absent	Present in some samples	Absent	Absent	Absent		Absent	Not reported	Not reported
Conceptacles in surface view	Usually slightly protruding, sometimes flush with surface	Flush to protruding, usually slightly protruding	Slightly or not protruding	Slightly or not protruding	Flush with surface	Flush with surface	Slightly or not protruding	Slightly protruding		Slightly or not protruding	Not reported	Protruding
Habit of lower side	Small lamellae or excrescences present; usually heavy coverage of sessile invertebrates	Small lamellae or excrescences present	Small lamellae present in some samples	Small lamellae present	A few lamellae present	Small lamellae present	Small lamellae present	Smooth, on drifted fishing net		Superimposed lamellae with excrescences; usually heavy coverage of sessile invertebrates	Not reported	Not reported
Thickness of thallus (μm)	470–1,800	200–750	415–2,500	600–750	412–1,700	260–301	450–850	250–280	N/A	500–950	Up to 4,000	200–250
Trichocytes	(+)	+	−	−	(+)	−	(+)	−	−	−	Not known	−
Height/width of hypothallial cells (μm)	4–18	4–20	7–20	4–15	5–15	5–15	4–16	17–23	8–16	5–10	10–20	3–9
Length of hypothallial cells (μm)	4.5–28	4–29	4–18	4–15	4–15	5–15	4–10	9–14	7–10	10–25	Not known	5–11
Height of perithallial cells (μm)	5.5–42.5	8–38	9–50	7.5–25	5–20	4–18	5–22	9–18	13–25	7–24	10–20 (–40)	6–10
Width of perithallial cells (μm)	3–20	4.5–22	7–21	9–18	5–16	6–20	3–14	7–14	8–13	5–11	6–8 (15)	5–9
Number of epithallial cells	1–2	1–2	1(–2)	1–2	1	1	1–2	1	1	1–2	Not known	1–2
Height of epithallial cells (μm)	1–7.5	0.5–7.5	1.5–7.5	4–7.5	2.5–6	3–8	1–6	2–5	2.5–4	1–3	Not known	2–3
Width of epithallial cells (μm)	3–14	2.5–15	6–28	9–15	6–14	8–14	5–11	8–10.5	10–12.5	2–11	Not known	5–7

[a]Characters of *L. lobatum* based on the description by Hamel and Lemoine (1953); characters of *L. searlesii* based on the description by Richards et al. (2018).
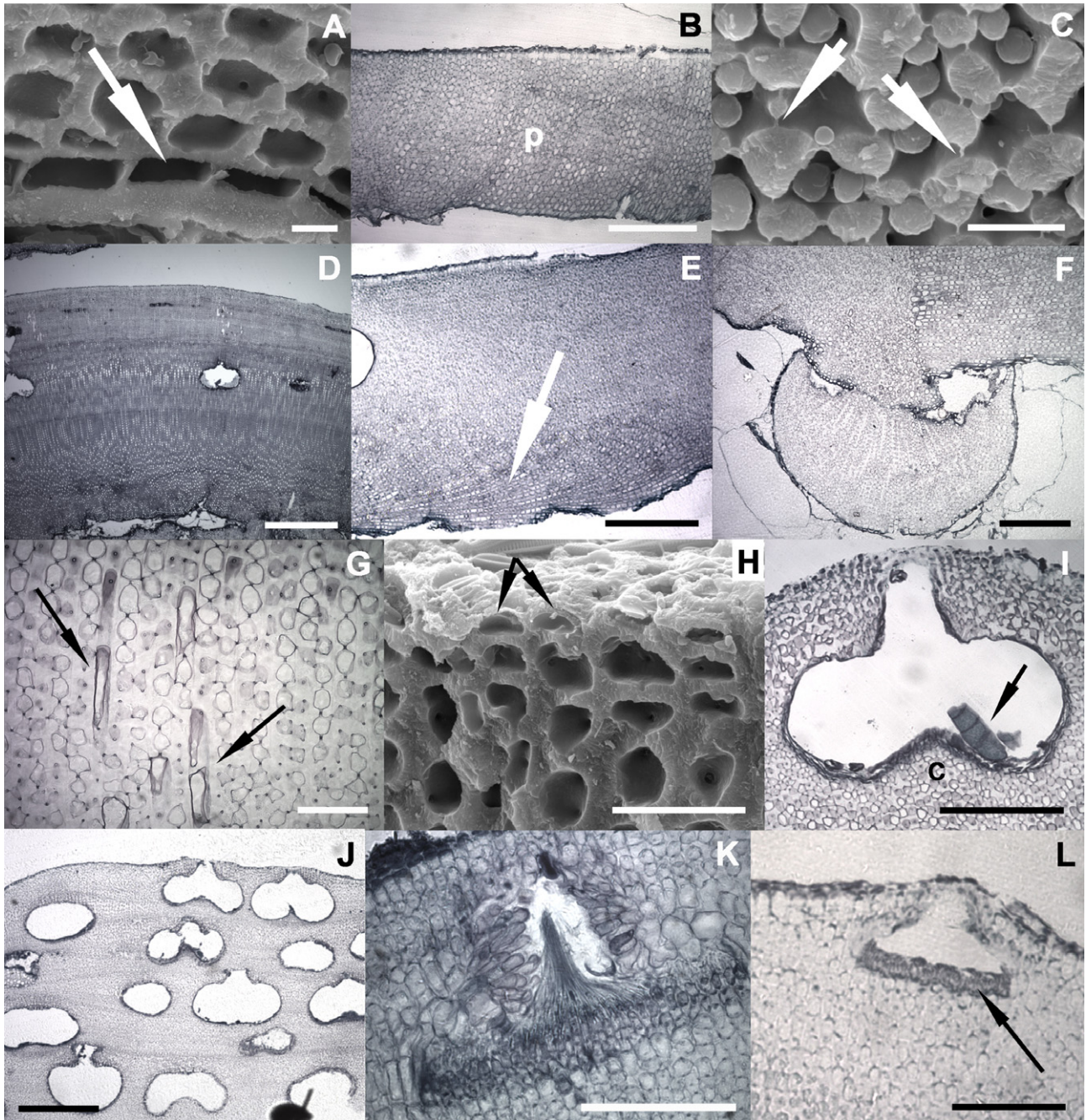
Fig. 7. *Lithophyllum stictiforme* complex: details of morpho-anatomy. (A) Detail of monostromatic hypothallus; arrow indicates hypothallial cell (scale bar: 10 μm). (B) Thallus cross-section showing hypothallus and perithallial filaments (p); sample Tremiti3-2014 (scale bar: 200 μm). (C) Perithallial filaments joined by secondary pit connections (arrows) (scale bar: 15 μm). (D) Cross section of thick thallus with some buried conceptacles; sample Trieste7 (scale bar: 500 μm). (E) Cross section showing perithallial filaments parallel to surface (arrow); sample Giannutri4 (scale bar: 300 μm). (F) Outgrowth issued from ventral side; sample Ponza29 (scale bar: 200 μm). (G) Detail of trichocytes (arrows); sample Tavolara8 (scale bar: 50 μm). (H) Epithallial cells (arrows) (scale bar: 20 μm). (I) Uniporate conceptacle with basal columella (c) and tetrasporangium (arrow); sample Ponza21 (scale bar: 200 μm). (J) Buried tetra/bisporangial conceptacles; sample Molara27 (scale bar: 300 μm). (K) Immature female gametangial conceptacle; sample Portofino4 (scale bar: 100 μm). (L) Male gametangial conceptacle with bottom covered by spermatangia (arrow); sample Tremiti1 (scale bar: 100 μm). [Color figure can be viewed at wile yonlinelibrary.com]

2011, 2013, Hind et al. 2014a, 2016, Mateo-Cid et al. 2014, Nelson et al. 2015, Hernandez-Kantun et al. 2016, Caragnano et al. 2018), which is sometimes striking and has been shown for the tropical reef consolidator *Porolithon onkodes* representing a complex of at least 20 cryptic species (Gabrielson et al. 2018). Based on our phylogenetic analyses of specimens with the *Lithophyllum stictiforme* morphology

along with support from GMYC and ABGD species delimitations and geographic distributions, we conclude that numerous distinct species are passing under *Lithophyllum stictiforme*. Using the most conservative species delimitation analysis (ABGD) of the most conservative marker in terms of substitution rate (*psb*A), we recognize 13 species. We based our species discrimination on *psb*A because this marker amplified the greatest number of samples and had the most complete phylogenetic coverage. Pairwise sequence divergences for *psb*A and *rbc*L also support recognition of multiple cryptic species. Pairwise divergences among our *psb*A sequences range mostly between 2% and 5% and are within the range considered indicative of species separation in other red algal genera (e.g., divergences as low as 0.88% in *Campylaephora*, 1% in *Griffithsia* and 0.95% in *Gracilaria*; Pezzolesi et al. 2017 and references therein). Within the coralline algae, five species of the geniculate genus *Bossiella* with overlapping distributions in the Northeast Pacific Ocean had pairwise divergences of ~0.9% for both *psb*A and *rbc*L (Hind et al. 2014a). In the current study, *rbc*L pairwise divergences among sequences in the *L. stictiforme* complex ranged mostly between 4% and 8%. As a comparison, cryptic species recognized by Gabrielson et al. (2018) in the *P. onkodes* complex differed by >1%, and divergences of 0.5%–0.8% were used to distinguish species in other genera of Corallinales (Gabrielson et al. 2011, Hind et al. 2016).

To assess whether the 13 species recognized within the *Lithophyllum stictiforme* complex can be distinguished based on morpho-anatomical features is a task that requires further detailed investigation and we prefer to leave that to future studies. As with most cryptic species, our morpho-anatomical observations showed a sizeable overlap between the clades for most of the characters considered (Table 1), making it difficult to suggest characters that can be easily used for identification purposes. However, we were able to obtain and examine in detail a large number of specimens only for the clades 1, 2, and 3. The numbers of specimens available for the other clades were too few, making detailed observations impossible (for several clades we could not even comment on reproductive characters). So, we do not exclude the possibility that species in the *L. stictiforme* complex can be recognized morpho-anatomically, perhaps based on different combinations of characters.

Differences in ecology or habitat might also be useful to distinguish species in this complex. The samples in this study were collected by many different operators and obtained mainly from coralligenous habitats. For many of the specimens, additional environmental information (e.g., exact depth, inclination of the substratum, type of surface colonized, exposure to wave motion/currents, occurrence on an exposed surface or within a crevice, associated species, temperatures, salinities, nutrients, etc.) was not provided, so that no strong conclusions can be drawn at this time. It is noteworthy that samples from clade 10 were collected in shaded crevices in the shallow subtidal zone, at ~−0.5 m depth and are clearly associated with a different depth compared to the other clades. In addition, only samples from clade 8 were growing on discarded fishing lines, but the natural substrata on which these algae normally grow are unknown. Ecological traits have received less attention than morpho-anatomical data in relation to species discrimination in corallines, but recent studies suggest that this aspect should receive more consideration. Hernandez-Kantun et al. (2015a) highlighted that the generitype *Lithophyllum incrustans* can be discriminated in the field from its close relatives *L. bathyporum* and *L. hibernicum* based on its subtidal habitat and its capacity to form rhodoliths. Gabrielson et al. (2018) remarked that cryptic species in the *Porolithon onkodes* complex occupy different habitats, characterized by different wave exposures, irradiances, and biotic interactions. These authors concluded that a number of evolutionary drivers such as eco-physiology, hydrodynamic regimes, and biotic interactions played a major role in the diversification of this complex. Like these studies, future investigations of the *L. stictiforme* complex will likely find ecological niche differences.

The formal description of the new lineages discovered in this study as new species will increase the number of *Lithophyllum* species currently recognized in the Mediterranean from 14 (Cormaci et al. 2017) to at least 26. Several of our clades appear to have a restricted geographic distribution and it can be expected that further collections in areas not yet sampled will lead to the discovery of new lineages recognizable as distinct species. This prediction is particularly true for the more eastern parts of the Mediterranean (e.g., Aegean Sea, Turkey, Levant states) and the African shores, from which we could not obtain any samples. Based on these considerations, our results support the view of Hernandez-Kantun et al. (2016) that in *Lithophyllum* species diversity is likely two to four times greater than currently recorded in each geographic region.

Nomenclature and application of Linnaean names to molecular lineages. A common difficulty posed by cryptic species identified by DNA sequencing is the correct application of Linnaean binomials. When Linnaean names are available for a complex of cryptic species, the only unambiguous solution is to obtain DNA sequences from the type specimens. This approach has often been successful in corallines and DNA sequences of sufficient quality and length for species identification have been obtained from many 19th and 20th century type specimens, allowing for the unambiguous applications of species names originally based on morpho-anatomy (Hernandez-Kantun et al. 2016, and references therein, Richards et al. 2017). Names that are available in the literature and that most probably pertain to Mediterranean members of the *Lithophyllum stictiforme* complex include *L. agariciformis*, *L. bermudense*,

L. cabiochiae, L. expansum f. *agariciforme, L. expansum* f. *exigua, L. expansum* f. *foliacea, L. expansum* f. *repens, L. grandiusculum, L. lobatum, L. frondosum,* and *L. giganteum* (including their basionyms and homotypic synonyms; Guiry and Guiry 2018). We obtained DNA sequences from the lectotype of *Melobesia stictiformis* and a syntype of *L. lobatum.* Athanasiadis (1999) designated as lectotype two fragments among the 12 that comprise the type collection of *M. stictiformis* in S. We obtained a partial *rbc*L sequence from one of the two fragments selected and illustrated by Athanasiadis (1999) (Fig. 1; the fragment marked by an arrow in our Fig. S1). Herein, we narrow the lectotype to this fragment as per Article 9.17 of the ICN (Turland et al. 2018).

Based on our results, *Lithophyllum stictiforme* is the name that applies to the most common subtidal *Lithophyllum* in the central Mediterranean and the main *Lithophyllum* contributing to coralligenous concretions. Most of the validly published names available for the *L. stictiforme* complex are based on basionyms more recent than *Melobesia stictiformis* (Areschoug in J. Agardh 1852). However, *L. giganteum* was described by Zanardini in 1843, and it therefore predates *M. stictiformis.* The description of *L. giganteum* is very brief (translated from Italian: "it grows at the bottom of the sea, and is truly unique for the giant shape of the compressed foliage," Zanardini 1843: 43). Several subsequent authors (Hauck 1885, De Toni 1905, Preda 1909, Hamel and Lemoine 1953), who perhaps saw original material of Zanardini, considered this species conspecific with *L. expansum* (the name used in the past for the most common *Lithophyllum* from coralligenous concretions, which we now know to collectively apply to the whole *L. stictiforme* complex). The epithet *giganteum* itself refers to the large size of the species, suggesting that this species is likely to belong to our clade 1. We enquired with museums and herbaria hosting the original Giovanni Zanardini collections (MCVE, FI, RO, Museo di Bassano del Grappa), but we could not locate any authentic specimens of *L. giganteum.* If the original Zanardini specimens are found and DNA sequences are obtained from them, it is possible that *L. giganteum* will become the earliest name available for the clade1 and therefore replace *L. stictiforme.* We also believe that *L. cabiochiae* is most probably conspecific with *L. stictiforme. Pseudolithophyllum cabiochiae,* basionym of the species, was described by Boudouresque and Verlaque (1978) (as "*cabiochae*") based on material from Pianottoli Caldarella (Corsica), collected between −4 and −5 m depth under a protrusion of a vertical rocky surface. Unfortunately, the type specimen has been stored in formalin and obtaining informative DNA sequences from it is highly unlikely. Boudouresque and Verlaque (1978), however, remarked that *P. cabiochiae* is distinctive for the purple-violet color and the constant presence of radial lines under the lower surface of the thallus, with concentric lines that are particularly evident

near the edge. Boudouresque and Verlaque (1978) also emphasized the large size of this species (20–25 cm in diameter) and its lobed margins, with proliferations that can develop underneath. All specimens that we sequenced in which these features were evident (e.g., Gallinara1, Portofino1, Ponza29, Ustica PG1, Ustica PG2, Otranto5) invariably belonged to clade 1.A definitive solution will require DNA sequences from new specimens collected from the type locality. Based on the present evidence, we agree with the decision of Cormaci et al. (2017) to consider *L. cabiochiae* conspecific with *L. stictiforme,* which has nomenclatural priority.

The other two names that we can assign here to clades of the *Lithophyllum stictiforme* complex are *L. lobatum* and *L. searlesii,* since sequences have been obtained from a syntype and holotype specimen respectively. The placement of these species in the *L. stictiforme* complex is noteworthy because these are the only non-Mediterranean lineages currently known in the complex. For a complete nomenclatural reassessment of the *L. stictiforme* complex, further studies attempting to sequence all relevant type specimens are needed.

Biogeography. Based on DNA-sequenced specimens, the distribution of the *Lithophyllum stictiforme* complex appears circumscribed to the North Atlantic Ocean and its diversity concentrated mostly in the Mediterranean. However, this might be a consequence of the scarcity of DNA sequences from non-Mediterranean samples. The only non-Mediterranean regions from which this complex can be presently confirmed are the Canary Islands (*L. lobatum*) and North Carolina, USA (*L. searlesii*). Morphology-based reports of *L. stictiforme* from New Zealand and Brazil, for which the taxonomic identity was assessed by DNA sequences (Broom et al. 2008, Tamega et al. 2014), were shown to be incorrect (Hernandez-Kantun et al. 2016). We also believe that records from Australia (Huisman 2000) and Tanzania (Oliveira et al. 2005) are unlikely to represent members of the complex. Literature records of *L. stictiforme* from Atlantic regions close to the Mediterranean, such as the Iberian Peninsula (Lugilde et al. 2016 and references therein), and Canary Islands, Madeira, and Salvage Islands (John et al. 2004) are likely to be correct and refer to members of the complex. Records from North Atlantic tropical regions, such as Senegal (John et al. 2004), Florida (Taylor 1960), Hispaniola (Betancourt and Herrera-Moreno 2001), Caribbean Mexico (Mendoza-Gonzalez et al. 2017), and Colombia (Diaz-Pulido and Diaz-Ruíz 2003) require confirmation based on DNA sequences.

In terms of evolutionary origin, a recent time-calibrated SSU rDNA phylogeny indicates that the ancestor of the *Lithophyllum stictiforme* lineage and its sister lineage (formed by *Lithophyllum incrustans* and *L. dentatum*), occurred between 18 and 8 mya (Rösler et al. 2017). This period corresponds to a

shift in the composition of the Mediterranean biota, with the decrease of the Tethyan biogeographic affinities after the Burdigalian closure of the eastern connection with the Indo-Pacific (~19 mya). This hypothesis agrees with the fossil record, as fossils reliably referable to the *Lithophyllum stictiforme* complex date back to the Upper Langhian (14.5 mya; Hrabovský et al. 2016) and span the Lower Tortonian (11.6–9 mya; Braga and Aguirre 2001), the Messinian (7.2–5.3 mya; Braga et al. 2009, Sola et al. 2013), the Pliocene (Nalin et al. 2010) and the Pleistocene (2.6–0.01 mya; Basso et al. 2007, Bracchi et al. 2014). The *L. stictiforme* lineage predating the Messinian suggests an explanation for the high diversity of the complex in the Mediterranean. During the Messinian Salinity Crisis (MSC, 5.96–5.33 Ma), the Mediterranean Sea was disconnected from the Atlantic and subdivided into a number of highly saline basins, undergoing repeated phases of desiccation with the deposition of kilometers-thick evaporites (Wade and Brown 2006, Roveri et al. 2014). It is believed that most of the Mediterranean marine species disappeared during this period, although some deep areas and some areas near large river deltas probably served as refugia and allowed the local persistence of a high diversity (Roveri et al. 2014, Calvo et al. 2015). It is plausible that populations from the *L. stictiforme* complex survived in such areas, and that their separation resulted in allopatric speciation with a number of cryptic species maintained after the MSC. This pattern has already been proposed for the genetic diversity detected in some benthic fishes (Huyse et al. 2004) and sessile mollusks (Calvo et al. 2015). Subsequent climatic events such as the Quaternary glaciations (2 mya to present), which also altered the Mediterranean coastline and partially separated different sectors, probably further contributed to the current diversity and distribution of the *L. stictiforme* complex. In this scenario, it can be expected that many species of the complex will be Mediterranean endemics with a restricted distribution, a possibility that is supported by our results. For example, several of our clades were recorded from restricted geographic areas such as clade 6 (Tremiti Islands, Adriatic Sea), clade 7 (central and southern Spain), clade 9 (Sicily), clade 10 (French Riviera), and clade 11 (Sardinia). Additional DNA sequences from other North Atlantic regions and other areas of the Mediterranean will be necessary to understand in depth the evolutionary origin and phylogenetic diversification of the complex.

Implications for conservation. The discovery that in the Mediterranean at least 11 species of *Lithophyllum* have been passing as one has implications for the conservation of coralligenous habitats. Coralligenous assemblages are well-known repositories of high species diversity (α-diversity, ~1,700 species reported by Ballesteros 2006) and are characterized by a high spatial heterogeneity (β-diversity, Cecchi

et al. 2014). Their ecological importance is widely recognized, and they are considered a "special habitat type" that requires monitoring for environmental status in European policies (Marine Strategy Framework Directive, 2008/56/EC). A recent study concluded that species diversity alone is inadequate as a basis for setting conservation goals for these assemblages and that additional information, based on phylogenetic diversity, is needed to preserve ecosystem function and coralligenous evolutionary history (Doxa et al. 2016). Our results demonstrate high species diversity in one of the most important algal bioconstructors of these habitats. At least 9 of the 11 cryptic species that we uncovered in the Mediterranean include samples collected from coralligenous assemblages. Based on our data, *L. stictiforme* (clade 1) is the most common *Lithophyllum* in these communities and is the only species in the complex that can be recognized quite easily, due to its large-sized thallus often formed by superimposed lamellae that bear lobed margins and violet-magenta color. In general, identification from a visual inspection in the field or from photographs is not possible for other species in the complex. Since identifications of subtidal *Lithophyllum* in the Mediterranean have been so far based on gross morphology and morpho-anatomy, the species diversity of *Lithophyllum* has most likely been underestimated. The restricted geographic distribution of some clades also suggests the possibility of confusion between species occurring in different areas, with consequent underestimation of β-diversity. These problems affect ecological assessments of coralligenous assemblages, which are usually based on photographic sampling methods. This approach is convenient since it allows rapid collections of a large number of replicates during fieldwork, but it is totally inadequate to describe the species diversity of *Lithophyllum.* Similar caveats can be extended to recent physiological studies that focused on Mediterranean subtidal *Lithophyllum.* In recent years, due to the high sensitivity of their calcified thalli, coralline algae have become a key subject in investigations focusing on the effects of climate-driven ocean acidification (Hurd 2015, McCoy and Kamenos 2015). Several studies examined growth and calcification in response to acidification and increased temperatures in Mediterranean *Lithophyllum* (Garrabou and Ballesteros 2000, Martin and Gattuso 2009, Martin et al. 2013a,b, Nash et al. 2016, Rodriguez-Prieto 2016). Specimens used in these studies were identified as *L. frondosum, L. cabiochiae,* or *L. stictiforme,* and their taxonomic identities should now be reassessed. Based on the color and habit of the material illustrated, the material used by Martin et al. (2013a,b; as *L. cabiochiae*) appears to be *L. stictiforme* (clade 1). Conversely, the thalli depicted by Garrabou and Ballesteros (2000) cannot be reliably identified. These investigations were based on numerous thalli collected from the field, and it is possible that

more than one species could have been tested. For the other studies cited, the absence of illustrated specimens does not allow for an even tentative species identification. In the light of our results, we strongly recommend future studies on Mediterranean corallines, including those with ecological, physiological, or mineralogical focus, deposit voucher specimens in public herbaria and provide identifications based on DNA sequences. When this is not possible and identifications must be based solely on morpho-anatomy, specimens with habits corresponding to the *Lithophyllum stictiforme* complex should be left without species-level identification (either *Lithophyllum* cf. *stictiforme* or *Lithophyllum* sp.).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Box A2338 in S, containing the lectotype material of *Melobesia stictiformis*. The lectotype is restricted here to the fragment indicated by the arrow, from which a partial *rbc*L sequence was obtained.

Figure S2. Phylogram inferred from PhyML analysis of the concatenated *psb*A-*cox*2,3 data set for the *Lithophyllum stictiforme* complex. Bootstrap support (BP) and Bayesian posterior probabilities (PP) indicated at nodes. Support values from left to right: BP for NJ, BP for RAxML, and Bayesian PP. BP values lower than 50% and PP lower than 0.8 not shown. Thick lines indicate full support (100% BP for both NJ and RAxML and 1.0 PP). Blue boxes indicate the clades corresponding to species recognized in the *psb*A phylogeny. Scale number indicates substitutions/site.

Figure S3. Phylogram inferred from PhyML analysis of the concatenated *psb*A-*rbc*L data set for the *Lithophyllum stictiforme* complex. Sequences obtained from type specimens are marked in bold. Bootstrap support (BP) and Bayesian posterior probabilities (PP) indicated at nodes. Support values from left to right: BP for NJ, BP for RAxML, and Bayesian PP. BP values lower than 50% and PP lower than 0.8 not shown. Thick lines indicate full support (100% BP for both NJ and RAxML and 1.0 PP). Blue boxes indicate the clades corresponding to species recognized in the *psb*A phylogeny. Scale number indicates substitutions/site.

Table S1. Collection and voucher details for the samples of *Lithophyllum* sequenced in the study.

Table S2. Details of additional sequences of *Lithophyllum* included in the molecular data sets used for the phylogenetic analyses.

Table S3. Summary of reproductive anatomical characters for the clades recovered in the molecular phylogenies of the *Lithophyllum stictiforme* complex.