

Assessment of coralline species diversity in the European coasts supported by sequencing of type material: the case study of *Lithophyllum nitorum* (Corallinales, Rhodophyta)

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Abstract – A constant effort in sequencing an extensive number of specimens originating from comprehensive sampling had return an unprecedented amount of information fostering our understanding of diversity, evolution and distribution of coralline algae; however, many sequences lack reliable assignation of a taxonomic name, specially at the species level. Recently, the sequencing of type material allowed to bridge this gap by providing a direct link between the DNA sequence and the type bearing name. For instance, in the genus *Lithophyllum*, the identity of three species, generally abundant along the European Atlantic and the Mediterranean, was demonstrated by including sequences of the type material. Nevertheless, for less conspicuous species, such as *Lithophyllum nitorum*, data are still needed to assess distribution, anatomy, phylogenetic affinities and taxonomic status. Using DNA sequences recovered from the type material of *L. nitorum*, further recent collections were resolved as conspecific and used to improve the description and refine the distribution of this species. *Lithophyllum nitorum* consisted of subtidal, thin crusts overgrowing fragments of dead maerl, pebbles and drifted fragments of fishing lines. The presence of uniporate conceptacles markedly protruding can be considered an external morphological feature useful to identify *L. nitorum* from other subtidal *Lithophyllum* species with similar habitat. *Lithophyllum nitorum* is reported for the Mediterranean Sea and confirmed in the North East Atlantic, a similar distribution as for *L. incrustans* and *L. hibernicum*.

Biodiversity / Corallinales / DNA barcoding / distribution / *Lithophyllum* / maerl beds / Mediterranean / North East Atlantic / systematics / type collections

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INTRODUCTION

Lithophyllum Philippi is considered one of the most speciose non-geniculate coralline algal genera with 130 specific/infraspecific names currently accepted (Guiry & Guiry, 2017). This genus is mainly distributed in tropical and temperate waters, where it contributes to the formation of characteristic calcareous bioconstructions such as the Caribbean algal ridges (Adey, 1978), the *coralligenous* and *trottoir* communities in the Mediterranean Sea (Ballesteros, 2006; Pezzolesi *et al.*, 2016), and singular maerl beds in the North East Atlantic (Peña *et al.*, 2013; Hernández-Kantún *et al.*, 2015). In this latter region, recent literature pointed out 12 species of *Lithophyllum* occurring under different growth-forms, from the intertidal to subtidal: the generitype *Lithophyllum incrustans* Philippi, together with *L. bathyporum* Athanasiadis & Ballantine, *L. byssoides* (Lamarck) Foslie, *L. crouaniorum* Foslie, *L. cystosirae* (Hauck) Heydrich, *L. decussatum* (Ellis & Solander) Philippi, *L. dentatum* (Kützing) Foslie, *L. hibernicum* Foslie, *L. nitorum* W.H.Adey & P.J.Adey, *L. orbiculatum* (Foslie) Foslie, *L. papillosum* (Zanardini *ex* Hauck) Foslie and *L. vickersiae* Me.Lemoine (Chamberlain & Irvine, 1994; Hernandez-Kantún *et al.*, 2015, reviewed in Lugalde *et al.*, 2016). So far, only three taxa – *L. incrustans*, *L. hibernicum* and *L. bathyporum* – have been subject of molecular studies using type material and recent collections from a wide geographic area (Hernandez-Kantun *et al.*, 2015). It allowed a reassessment of the phylogenetic affinities of these species, and the consideration of earlier collections identified as *L. duckerae* (Cornwall), *L. fasciculatum* and *L. dentatum* (Ireland and Britany) as *L. hibernicum* and *L. incrustans*, respectively. These results also demonstrated that *L. hibernicum* and *L. incrustans* have a high morphological plasticity, for which features mainly related with epithallial cells and sporangial conceptacles were useful for their identification (Hernandez-Kantun *et al.*, 2015, Vázquez & Peña, 2016). A fourth species – *L. byssoides* – has been also reassessed based on Atlantic and Mediterranean collections; however, molecular information from the lectotype (*Nullipora byssoides* Lamarck) is still pending to be obtained for a better clarification (Pezzolesi *et al.*, 2016).

With regards to the habitat, while the crustose *Lithophyllum hibernicum* (as *L. incrustans* in the literature before 2015) and *L. orbiculatum* are common in the intertidal, and *L. crouaniorum* in the subtidal (Adey & Adey, 1973; Chamberlain *et al.*, 1988, 1991; Chamberlain & Irvine, 1994), *L. nitorum* is considered a rare species, occurring on rock and shells only in the sublittoral zone down to 46 m (Adey & Adey, 1973; Chamberlain & Irvine, 1994). *Lithophyllum nitorum* was described by Adey & Adey (1973), and it was mainly characterized by small, adherent crusts relatively thin, with orange- pink colour, a smooth surface without orbital ridges, highly to somewhat glossy, a single layer of epithallial cells (rarely two), short subepithallial cells, cortical cells of contiguous filaments usually laterally aligned, conceptacles markedly protruding, usually bisporangial with a pore canal tapering to the surface and lined by small papillae, and sporangial chambers not globular with a small columella (Adey & Adey, 1973; Chamberlain & Irvine, 1994). In addition, Chamberlain & Irvine (1994) pointed out the absence of secondary growth and growth over *Lithothamnion sonderi* Hauck as key characters to identify plants of *L. nitorum* in the British Isles. The type locality of *L. nitorum* is Port Erin Harbour (Isle of Man, 3-9 m depth, as epilithic in Adey & Adey, 1973), but it was also reported southwards to northern Spain (Chamberlain & Irvine, 1994); in fact, Adey & Adey (1973) recorded *L. nitorum* at most of the Bay of Biscay stations

sampled, reaching 24% abundance at 37-46 m off Castro Urdiales (Cantabrian Sea) compared to 14% between 8-30 m in the British Isles, particularly abundant off the Lizard (Cornwall) while it occurs sporadically northwards (*ca.* 0.2-0.7% mean abundance, fig. 53 in Adey & Adey, 1973). In the Mediterranean Spain, Braga & Aguirre (1995) observed plants attributed to *L. nitorum* in Almería and in Cabrera (Balearic Islands), and reported it in southern Spain as minor component of fossil coralline associations in the Neogene (Miocene and Pliocene, *ca.* 23 million years ago).

With the aim of re-assessing coralline species diversity and distributions along the European coasts, the sequencing of type specimens coupled with extensive sampling across a wide geographic area became the most efficient practice to refine the concept of a species (Puillandre *et al.*, 2011). In the present study, we obtained DNA sequences (*psbA*) from the holotype of *Lithophyllum nitorum*, and we inferred species delimitation among recent collections made along the European coasts to assess the distribution of this species.

MATERIALS AND METHODS

Collections studied

We examined the holotype of *Lithophyllum nitorum*, collected in Port Erin Harbour, Isle of Man, at 3-9 m depth (Adey & Adey, 1973), and preserved in the herbarium of the Smithsonian Institution (US 70-10B, 10-30, Fig. 1). In addition, we studied eleven specimens identified as *Lithophyllum* sp. collected in recent subtidal surveys in Brittany (Trevignon, Fig. 2), Galicia (Ons and Cíes archipelagos, Islas Atlánticas National Park, Figs 3-7), and Mediterranean Spain (Granada, Figs 8-9) (Table 1). The Atlantic collections corresponded to thin crusts overgrowing dead maerl and pebbles associated to maerl beds between 11-15 m depth in 2011 (Figs 2-7), while the Mediterranean specimens consisted on crusts on a pebble and a drifted fragment of a fishing line at 15 m depth in 2012 (Figs 8-9). Recent collections were air-dried or oven-dried (50°C), preserved in plastic bags with silica gel before molecular and morphological studies, and deposited in the herbaria of Universidade de Santiago de Compostela (SANT) and Museum National d'Histoire Naturelle (PC) (acronyms follow Thiers, 2015).

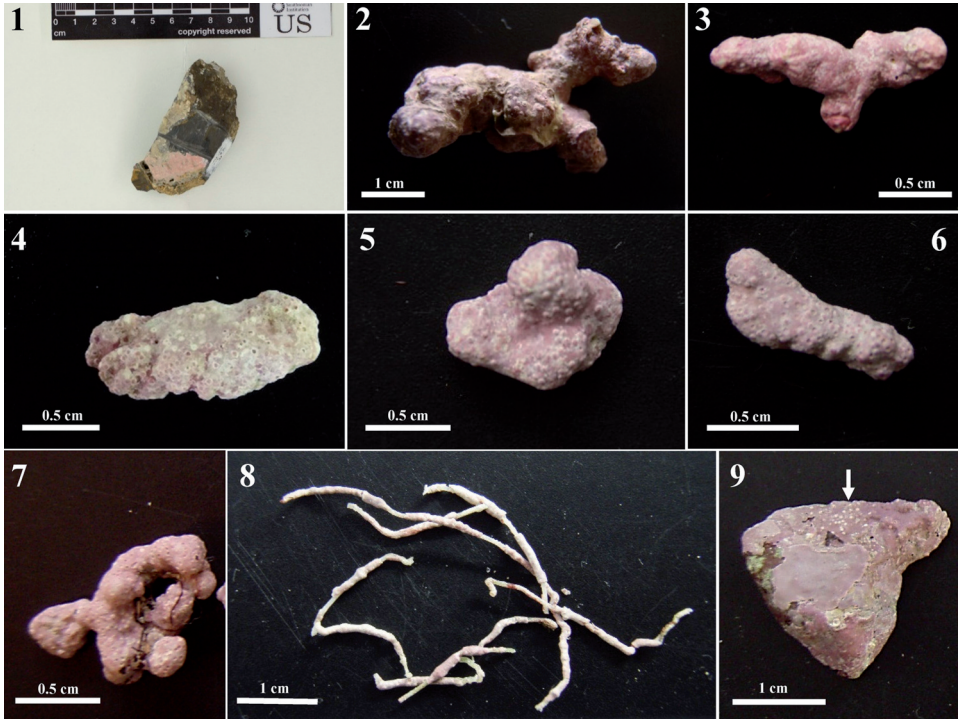
DNA sequencing and phylogenetic analyses

Eleven specimens from recent subtidal collections were cleaned under a stereomicroscope and selected surfaces were ground for DNA extraction. Genomic DNA was extracted using a NucleoSpin® 96 Tissue kit (Macherey-Nagel, GmbH and Co. KG, Germany), and DNeasy® Blood & Tissue Kit (Qiagen, Valencia, CA) following manufacturer's recommendations. DNA extractions and amplifications of the holotype of *Lithophyllum nitorum* housed in US were performed separately from recent collections and with negative controls run throughout at Smithsonian Laboratories of Analytical Biology, Washington DC, US; extraction of total genomic DNA of the holotype followed the modified protocol by Broom *et al.* (2008) of the Qiagen DNeasy Blood and Tissue Kit® (Qiagen, Crawley, UK). The mitochondrial

Table 1. Sample information for species included in molecular analyses. In bold, holotype specimen of *Lithophyllum nitorum*. Additional COI-5P and *psbA* sequences downloaded from GenBank are detailed

Species	Voucher	GenBank accession number (COI-5P)	GenBank accession number (<i>psbA</i>)	Collection details	Herbarium
<i>Lithophyllum nitorum</i>	US 70-10B		MG648773	Port Erin Harbour, Isle of Man, UK (Adey & Adey, 1973)	US 70-10B
<i>L. nitorum</i>	CPVP913	MG648771		Subtidal (15 m), maerl bed, Le Dragon, Trevignon, France, 13/05/2011. Coll. W. Thomas, Y. Fontana.	SANT-Algae 31427
<i>L. nitorum</i>	CPVP760	MG648763		Subtidal (13 m), maerl bed, Ons, Ria de Pontevedra, Galicia, Spain, 07/04/2011. Coll. I. Bárbara, R. Barreiro, V. Peña.	SANT-Algae 31420
<i>L. nitorum</i>	CPVP765	MG648764		Subtidal (13 m), maerl bed, Ons, Ria de Pontevedra, Galicia, Spain, 07/04/2011. Coll. I. Bárbara, R. Barreiro, V. Peña.	SANT-Algae 31421
<i>L. nitorum</i>	CPVP772	MG648765		Subtidal (13 m), maerl bed, Ons, Ria de Pontevedra, Galicia, Spain, 07/04/2011. Coll. I. Bárbara, R. Barreiro, V. Peña.	SANT-Algae 31422
<i>L. nitorum</i>	CPVP774	MG648767		Subtidal (13 m), maerl bed, Ons, Ria de Pontevedra, Galicia, Spain, 07/04/2011. Coll. I. Bárbara, R. Barreiro, V. Peña.	SANT-Algae 31423
<i>L. nitorum</i>	VPF00474	MG648769		Subtidal (13 m), maerl bed, Ons, Ria de Pontevedra, Galicia, Spain, 07/04/2011. Coll. I. Bárbara, R. Barreiro, V. Peña.	SANT-Algae 31424
<i>L. nitorum</i>	VPF00475	MG648762		Subtidal (13 m), maerl bed, Ons, Ria de Pontevedra, Galicia, Spain, 07/04/2011. Coll. I. Bárbara, R. Barreiro, V. Peña.	SANT-Algae 31425
<i>L. nitorum</i>	VPF00482	MG648766	MG648774	Subtidal (13 m), maerl bed, Ons, Ria de Pontevedra, Galicia, Spain, 07/04/2011. Coll. I. Bárbara, R. Barreiro, V. Peña.	SANT-Algae 31426
<i>L. nitorum</i>	VPF00388	MG648770		Subtidal (11 m), maerl bed, Cies, Ria de Vigo, Galicia, Spain, 07/04/2011. Coll. I. Bárbara, R. Barreiro, V. Peña.	SANT-Algae 31428
<i>L. nitorum</i>	LLG45961	MG648768		Subtidal (15 m), La Herradura, Granada, Spain, 19/09/2012. Coll. Le Gall, L., V. Peña.	PC0144138
<i>L. nitorum</i>	LLG4608A	MG648772		Subtidal (15 m), La Herradura, Granada, Spain, 19/09/2012. Coll. Le Gall, L., V. Peña.	PC0144151

Species	GenBank accession number (COI-5P)	GenBank accession number (psb4)	Collection details
Additional sequences analysed			
<i>Lithophyllum bathyporum</i>	–	KR736255	Brittany, Atlantic France
<i>L. bathyporum</i>	KR733435	–	Galicia, Atlantic Spain
<i>Lithophyllum byssoides</i>	–	JQ896252	Cádiz, Atlantic Spain
<i>L. byssoides</i>	–	JQ896251	Galicia, Atlantic Spain
<i>Lithophyllum dentatum</i> (Kützing) Foslie	KR708623	KR708611	Sitges, Mediterranean Spain
<i>Lithophyllum hibernicum</i>	KR708622	KR708594	Ireland
<i>L. hibernicum</i>	–	KR708614	Cornwall, UK
<i>L. hibernicum</i>	KR733526	–	Brittany, Atlantic France
<i>L. hibernicum</i>	KR733530	KR736256	Galicia, Atlantic Spain
<i>L. hibernicum</i>	KR733464	–	Port de Sète, Mediterranean France
<i>L. hibernicum</i>	KR733463	–	Granada, Mediterranean Spain
<i>Lithophyllum impressum</i>	–	JQ917416	NE Pacific
<i>Lithophyllum incrustans</i>	KR708619	JQ896237	Ireland
<i>L. incrustans</i>	KR708620	JQ896238	Brittany, Atlantic France
<i>L. incrustans</i>	KR733470	–	Galicia, Atlantic Spain
<i>L. incrustans</i>	KR733480	–	Granada, Mediterranean Spain
<i>L. incrustans</i>	–	KR708602	Granada, Mediterranean Spain
<i>Lithophyllum orbiculatum</i>	KR708625	KR708609	Brittany, Atlantic France
<i>Lithophyllum pustulatum</i> (J.V.Lamouroux) Foslie	–	KM407551	Mediterranean Spain
<i>Lithophyllum stictaeforme</i> (J.E. Areschoug) Hauck	–	KM407553	Mediterranean Spain
<i>L. stictaeforme</i>	–	KM407554	Mediterranean Spain
<i>Lithophyllum</i> sp.	–	KJ418414	Louisiana, USA
<i>Lithophyllum</i> sp.	–	KJ418415	Louisiana, USA
<i>Lithophyllum</i> sp3	MF133359	–	Galicia, Atlantic Spain
<i>Porolithon onkodes</i> (Heydrich) Foslie	GQ917289	–	New Caledonia
<i>Spongites agulhensis</i> Maneveldt, E. van der Merwe & P.W.Gabrielsons	–	KT184812	South Africa
<i>Spongites hyperellus</i> (Foslie) D.L.Pentose	–	GQ917495	Australia
Uncultured Corallinales	–	GQ917452	Vanuatu



Figs 1-9. *Lithophyllum nitorum*. **1.** Holotype specimen (voucher US 70-10B). **2, 3, 5-7.** Crustoses specimens overgrowing dead maerl (vouchers SANT-Algae 31427, 31426, 31424, 31425 and 31428, respectively). **4.** Crustose specimen overgrowing pebble (voucher SANT-Algae 31421). **8.** Crustose specimen overgrowing fragment of fishing line (voucher PC0144138). **9.** Crustose specimen overgrowing cobble – arrow – (voucher PC0144151).

COI-5P fragment was PCR-amplified using primer pairs GazF1/GazR1 (Saunders, 2005). The chloroplast *psbA* gene was amplified using primer pairs *psbA*-F1/*psbA*-R2 or *psbA*-F1/*psbA*600R (Yoon *et al.*, 2002), this latter used for the holotype. PCR reactions, thermal profiles, cleaning for sequencing and sequencing of the resulting products of the holotype followed Adey *et al.* (2015). Thermal profiles for PCR amplification of COI-5P and *psbA* fragments of recent collections followed Saunders & McDevit (2012) and Bittner (2009), respectively; PCR reactions followed Peña *et al.* (2015a). Excess primers and nucleotides from PCR products (labeled CPVP, Table 1) were removed with shrimp alkaline phosphatase and exonuclease I enzymes, and sequenced using the Sanger method at Macrogen facilities (<http://www.macrogen.com>). PCR products labelled VPF and LLG (Table 1) were purified and sequenced by Genoscope (Bibliothèque du Vivant program, Centre National de Séquençage, France). Sequences were assembled and aligned with the assistance of CodonCode Aligner® (CodonCode Corporation, USA) and adjusted by eye using SeaView version 4 (Gouy *et al.*, 2010). Sequences were submitted to the Barcode of Life Data Systems (project 'NGCOR', BOLD, <http://www.boldsystems.org>; Ratnasingham & Hebert, 2007) and GenBank (accession numbers listed in Table 1). Our analyses also comprised publicly available GenBank sequences of Atlantic and Mediterranean *Lithophyllum* spp., including those conspecific with type

collections of the generitype *L. incrustans*, *L. bathyporum* and *L. hibernicum* (Hernández-Kantun *et al.*, 2015) as well as the most relevant matches found with *L. nitorum* (> 90% similarity). These databases also encompassed sequences assigned to the genus *Lithophyllum*, and collected as associated crusts in Galician and Brittany maerl beds (Pardo *et al.*, 2017). According to recent phylogenetic studies (Van der Merwe *et al.*, 2015; Rösler *et al.*, 2016), publicly available sequences for *Spongites* spp. corresponding to the “Southern Hemisphere group” were selected as outgroups for the *psbA* alignment; while sequences of *Porolithon onkodes* were selected as outgroups for the COI-5P alignment (Table 1).

The alignments comprised 25 COI-5P and 22 *psbA* sequences. Phylogenetic relationships were inferred using Maximum Likelihood (ML) and Bayesian inference (BI) using Mega 6 and MrBayes 3.2.1, respectively (Ronquist & Huelsenbeck, 2003; Tamura *et al.*, 2013). Models of sequence evolution were estimated using the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC) obtained in jModeltest 2.1.3 (Darriba *et al.*, 2012). Maximum Likelihood and Bayesian analyses for the COI-5P and *psbA* alignments were performed under a generalized time-reversible with gamma + invariant sites heterogeneity model (GTR + G + I). The Bayesian analyses were performed under the same model with four Markov Chain Monte Carlo for 10 million generations, and tree sampling every 1000 generations.

Morphological studies of DNA-sequenced specimens

According to the previous phylogenetic analyses, representative specimens resolved conspecific to *Lithophyllum nitorum* were selected for anatomical examination with a scanning electron microscope (SEM, model JEOL JSM 6400, Universidade da Coruña, Spain). The examined specimens were deposited in the herbarium of Universidade de Santiago de Compostela (SANT). Cell length is the distance between primary pit-connections, and cell diameter is the measurement taken perpendicularly to this across the middle of the cell lumen. Conceptacle measurements were taken according to Adey & Adey (1973) and Chamberlain & Irvine (1994).

RESULTS

The holotype of *Lithophyllum nitorum* was successfully sequenced only for the plastid *psbA* gene (fragment of 450 bp), but it did not result in any match in GenBank database obtaining the highest similarity (94%) with *Lithophyllum impressum* from NE Pacific and Uncultured Corallinales (GQ917452) from Vanuatu (Table 1, Fig. 10; searched on 9 November 2017). Our phylogenetic analyses of the *psbA* alignment (22 sequences ranging from 450 to 822 bp, with 222 variable sites) resolved this species, albeit with low support values, as the sister taxa of *L. byssoides*. Moreover, this phylogeny recovered a recent collection obtained from a Galician maerl bed (voucher SANT-Algae 31426, Ons archipelago) with high similarity with the holotype of *L. nitorum* (0.07 % divergence, 3 bp difference, Fig. 10). Based on this recent collection, the phylogenetic tree obtained from the ML analysis of the COI-5P alignment (25 sequences resulting 17 haplotypes, ranging from 445 to

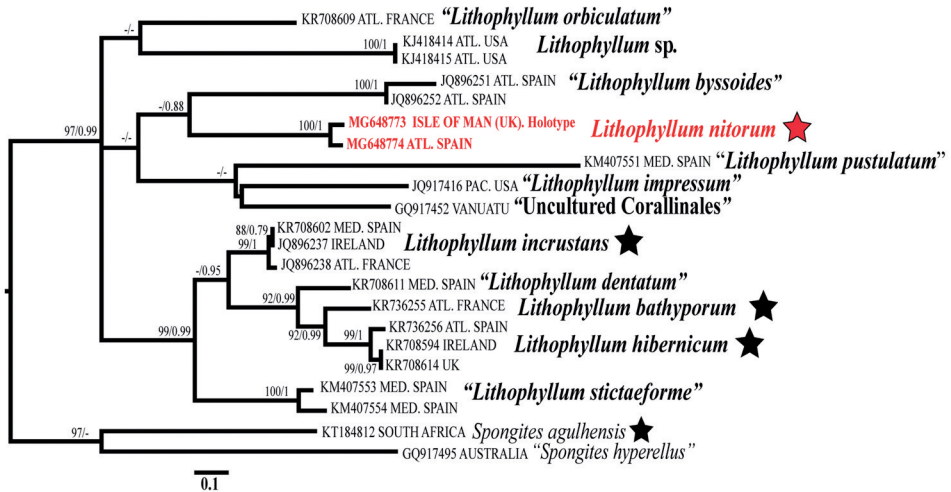


Fig. 10. Phylogenetic tree inferred from ML and BI analyses of *psbA* sequences of *Lithophyllum* recorded from European coasts, and highest similarity matches found in GenBank (> 94%). In red colour, *Lithophyllum nitorum* represented by holotype specimen and a recent collection. Stars indicate species names linked to type specimens by DNA sequences; quotation marks for species names based on morpho-anatomy or from topotype material (i.e. *L. impressum*). Bootstrap ML values > 60% and posterior probabilities > 0.60 from Bayesian inference are shown for each node. *Spongites agulhensis* and *S. hyperellus* from the Southern Hemisphere were used as outgroup.

664 bp in length, consisting of 216 variable sites) resolved another ten additional specimens (grouped into four haplotypes) from Atlantic and Mediterranean waters as conspecific with it (Fig. 11). Thus, our results confirm the occurrence of *L. nitorum* along the Atlantic European coasts of Brittany and the Northwest of the Iberian Peninsula (Galicia), and they also expand its distribution range southwards to the Mediterranean Spain (Granada).

The recent collections resolved conspecific with the holotype of *Lithophyllum nitorum* (Fig. 1) and consisted of small crusts overgrowing fragments of dead mael (*ca* < 3 cm), pebbles, cobbles, and a drifted fragment of fishing line (Figs 2-9). In contrast with the colour features provided in the literature for *L. nitorum*, the colour of our specimens was dark to pale pink, and the surface was somewhat glossy only in some specimens (i.e. Fig. 3). Uniporate conceptacles were conspicuously protruding (Figs 12, 13) and surrounded by a white rim (i.e. Figs 5, 12). The SEM examination of several specimens showed very thin crusts, < 500 μ m thick, very adherent to the substrate with a monomeric construction and non-coaxial medulla (Fig. 14). Epithallial cells were mostly flattened, 1-2 μ m tall \times 7-8 μ m diameter, disposed in 1-2 layers. Subepithallial cells were 5-8 μ m tall \times 3-10 μ m diameter, as short as or shorter than cells subtending them (Figs 16, 17). Cortical cells of contiguous filaments were laterally aligned, 6-14 μ m tall \times 5-8 μ m diameter, containing inclusions usually interpreted as floridean starch (Figs 15-17). Secondary pit-connections were observed; cell fusions were absent (Fig. 15). Uniporate conceptacles were protruding (Fig. 18); old conceptacles were buried, not infilled, with elliptical chambers of 54-100 μ m in height \times 180-290 μ m in diameter (Fig. 22). According to the shape of the chamber, some buried and empty conceptacles in

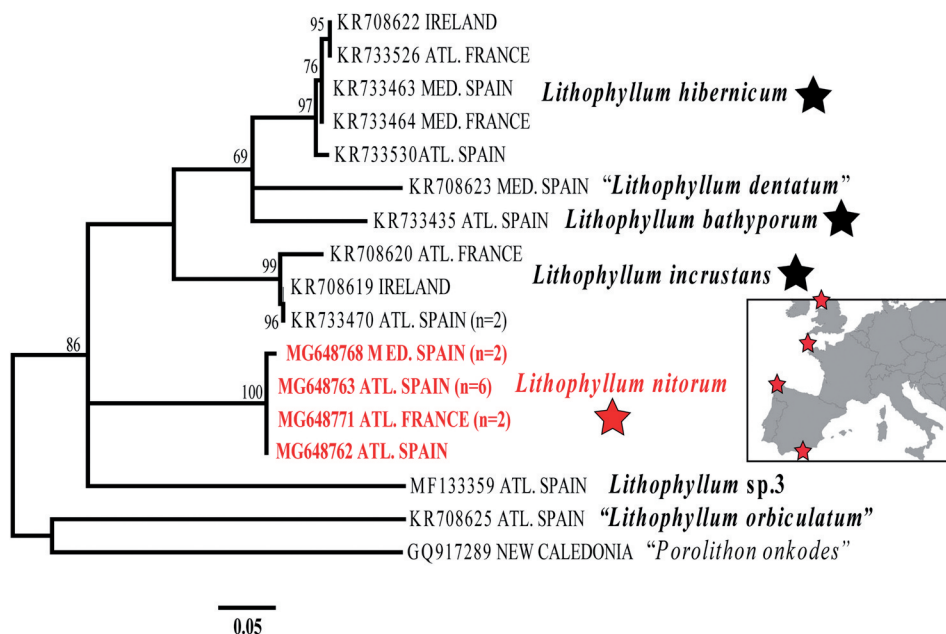
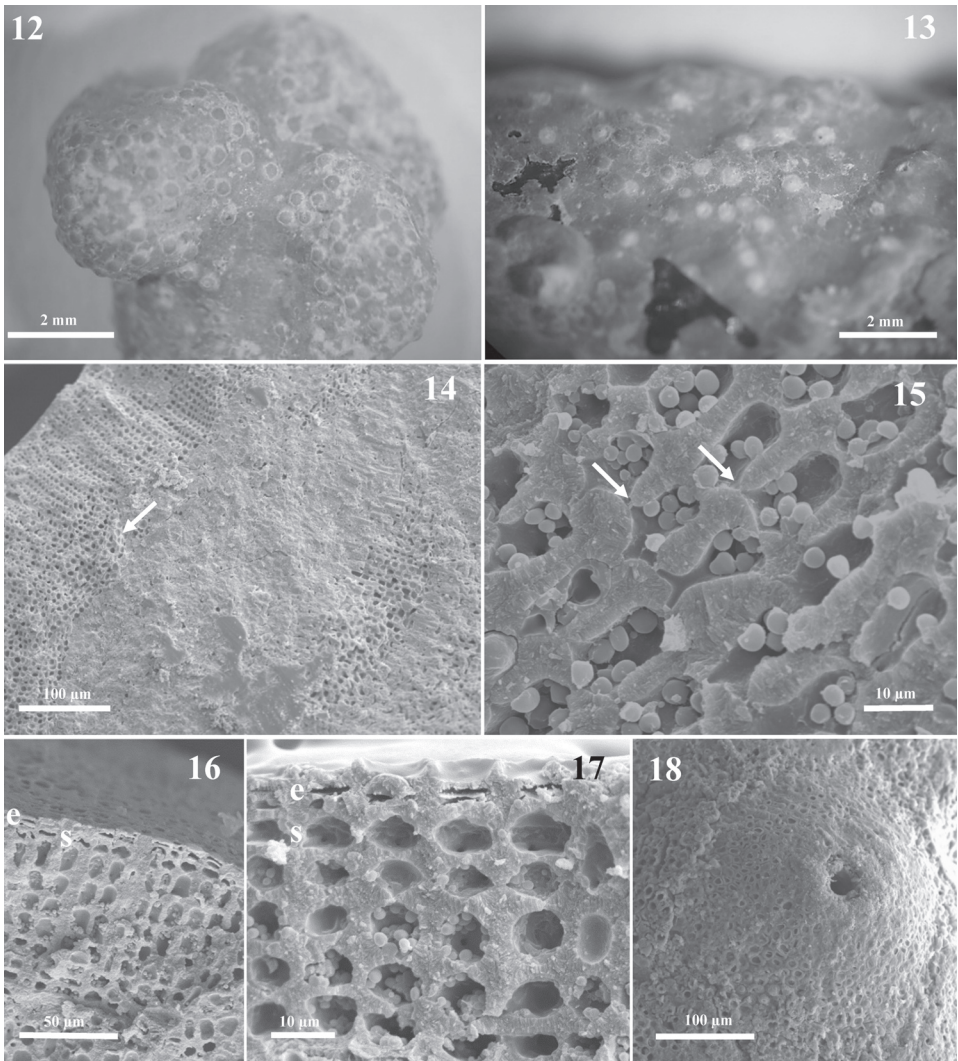


Fig. 11. ML tree inferred from DNA barcode (COI-5P). In red colour, recent collections resolved conspecific with the holotype of *Lithophyllum nitorum*, and their geographical location in the European coasts. Stars indicate species names linked to type specimens by DNA sequences; quotation marks for species names based on morpho-anatomy. Bootstrap values > 60 % are shown for each node. *Porolithon onkodes* (subfamily Metagoniolihoideae, Corallinales) was used as outgroup.

one plant collected in Ons archipelago (Galicia, voucher SANT-Algae 31426) corresponded to spermatangial conceptacles with chambers 21-22 μm in height \times 84-87 μm in diameter (Fig. 19). While another recent collection from the same Galician locality (voucher SANT-Algae 31421) showed an uniporate tetrasporangial conceptacle protruding (Fig. 20), with pore canal of 50 μm in length, tapering towards the surface (from 60 μm to 20 μm diameter) (Fig. 21); the chamber was 150 μm in height \times 250 μm in diameter, with a small calcified columella and tetrasporangia elongate, 117 μm in height \times 24 μm in diameter, zonately divided (Fig. 20).

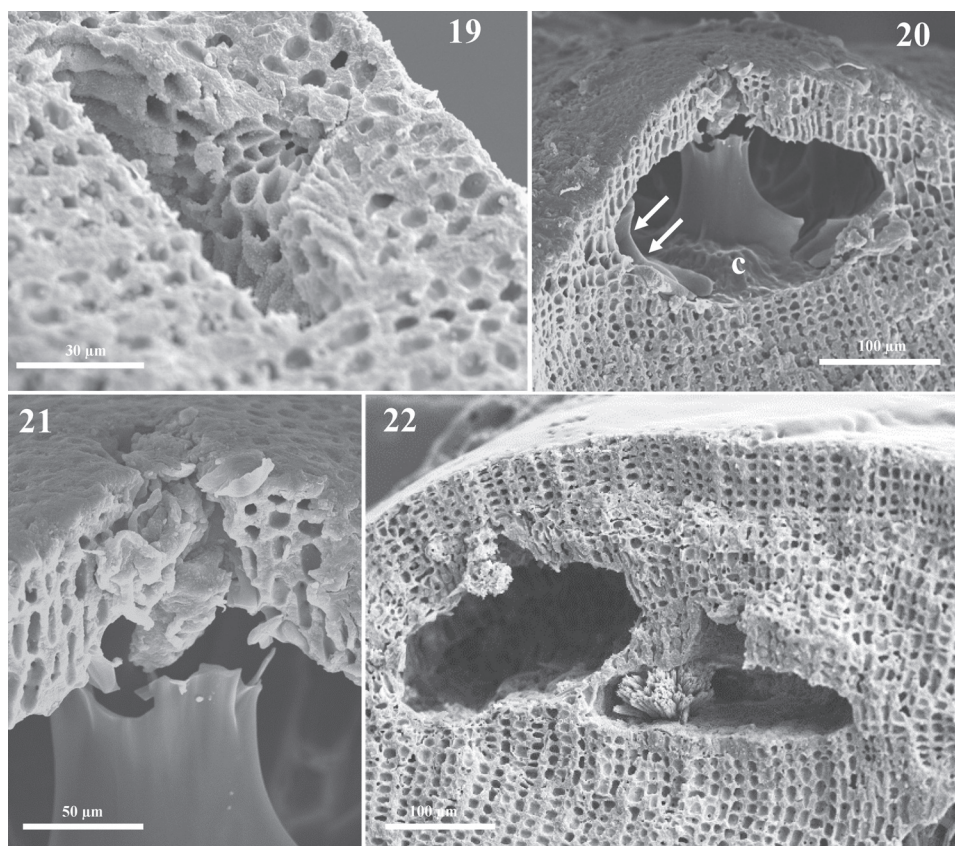
DISCUSSION

Based on a phylogenetic analysis of *psbA* sequences including the sequence obtained for the holotype of *Lithophyllum nitorum*, we were able to identify conspecific specimens in our recent collections from Atlantic and Mediterranean coasts (Fig. 11). However, none of the publicly available sequences from recent studies in Atlantic and Mediterranean *Lithophyllum* taxa (i.e. Hernández-Kantún *et al.*, 2015; Pezzolesi *et al.*, 2016; Pardo *et al.*, 2017) matched sequences from



Figs 12-18. *Lithophyllum nitorum*. **12-13.** Surface of specimens bearing some markedly protruding uniporate conceptacles and with white rims (vouchers SANT-Algae 31424, PC0144151). **14.** Vertical section showing adherent crust with monomerous construction –arrow- (voucher SANT-Algae 31426). **15.** Vertical section showing cortical cells of contiguous filaments laterally aligned, and joined by secondary pit connections –arrows-; lumen cells containing inclusions usually interpreted as floridean starch (voucher SANT-Algae 31421). **16-17.** Vertical section showing 1-2 layers of mostly flattened epithallial cells (e), and subepithallial cells (s) as short as or shorter than cells subtending them (voucher SANT-Algae 31428). **18.** Surface view showing uniporate conceptacles markedly protruding (voucher SANT-Algae 31422).

L. nitorum. According to the literature (Adey & Adey, 1973; Chamberlain & Irvine, 1994), *Lithophyllum nitorum* is considered a rare, subtidal species distributed from the British Isles to northern Spain, and recorded on rocks and shells. Our present study based on DNA sequence data confirmed the presence of *Lithophyllum nitorum*



Figs 19-22. *Lithophyllum nitorum*. **19.** Vertical section showing likely spermatangial uniporate conceptacle, empty and buried (voucher SANT-Algae 31426). **20.** Vertical section showing uniporate sporangial conceptacle containing tetrasporangia zonately divided – arrows – and small columella – c – (voucher SANT-Algae 31421). **21.** Detail of pore canal of uniporate tetrasporangial conceptacle tapering to surface (voucher SANT-Algae 31421). **22.** Vertical section showing old, empty uniporate conceptacles buried and not infilled (voucher SANT-Algae 31428).

in Brittany, the Northwest of the Iberian Peninsula (Galicia) and Mediterranean Spain. Furthermore, this study revealed the presence of this species on other substrates such as dead maerl, small pebbles or even drifted fragments of fishing lines. It is noteworthy to remark that all specimens collected are of small size and do not develop into large, conspicuous crusts; therefore, *L. nitorum* is likely to be easily overlooked. In agreement with the literature reports (Adey & Adey, 1973; Chamberlain & Irvine, 1994) and our morphological observations, the presence of uniporate conceptacles markedly protruding can be considered an external morphological feature useful to identify *L. nitorum* from other *Lithophyllum* species that also occur in the subtidal, such as *L. crouaniorum*, *L. bathyporum*, *L. hibernicum* or *L. incrustans* (Chamberlain & Irvine, 1994; Hernández-Kantún *et al.*, 2015). On the other hand, *L. nitorum* is described in the literature as orange- pink crusts, highly to somewhat glossy (Adey & Adey, 1973; Chamberlain & Irvine, 1994) while in our collections the colour varied from dark to pale pink and only some specimens were

somewhat glossy. Most of our specimens of *L. nitorum* have empty uniporate conceptacles, but one Galician plant is considered a male gametophyte based on the shape of spermatangial conceptacles. Chamberlain & Irvine (1994) pointed out that gametangial plants are unknown in the British Isles; Adey & Adey (1973, Table XII) recorded spermatangial conceptacles, however collection details are not indicated. According to the measurements provided in Adey & Adey (1973), the Galician specimen had smaller chambers (21-22 μm in height \times 84-87 μm in diameter vs. 22 μm in height \times 90-118 μm in diameter). In our study, only one Galician specimen examined showed tetrasporangial conceptacles that are in agreement with the literature (chamber 150 μm in height \times 250 μm in diameter vs 100-135 \times 255-385 μm and 94-156 μm \times 245-385 μm in Adey & Adey, 1973 and Chamberlain & Irvine, 1994, respectively). For the British Isles, Chamberlain & Irvine (1994) reported only bisporangial conceptacles, while Adey & Adey (1973) indicated that tetrasporangia were rare in the distribution range hitherto known for *L. nitorum* along the Atlantic European coasts. Likewise, we observed buried conceptacles not infilled which has not been reported earlier (Adey & Adey, 1973; Chamberlain & Irvine, 1994). The distribution range hitherto reported for *L. nitorum* is restricted from the British Isles to northern Spain (Adey & Adey, 1973; Chamberlain & Irvine, 1994); our results confirm records for Brittany and Galicia (Northwest of Spain), and they also expand the distribution of the species southwards to the Spanish coast of the Mediterranean Sea (Granada). Our Mediterranean record based on molecular evidences are congruent with previous observations of extant plants and Neogene fossil records in southern Spain and Balearic Islands (Braga & Aguirre, 1995). All recent collections assigned to *L. nitorum* were subtidal, made between 11-15 m, in agreement with the wide depth range provided in the literature (3-30 m in the British Isles, down to 46 m in northern Spain, Adey & Adey, 1973; Chamberlain & Irvine, 1994). The Atlantic collections from Brittany and Galicia were found on dead maerl or small pebbles associated with maerl beds. A recent DNA barcoding study carried out on maerl beds from these two Atlantic regions (Pardo *et al.*, 2017) revealed higher species diversity in associated crustose coralline algae than in maerl-forming species (9 vs 3, as total taxa obtained for each growth-form); two out of the nine crustose taxa corresponded to *Lithophyllum hibernicum* (Brittany) and an unidentified *Lithophyllum* sp.3 (Galicia) overgrowing pebbles, both with uniporate conceptacles not protruding (Fig. 3 in Pardo *et al.*, 2017). The record of *L. nitorum* as an additional encrusting species associated with maerl beds in Brittany and Galicia increases the associated diversity reported for this coastal habitat in the North East Atlantic (Peña *et al.*, 2014a).

The present work confirms the effectiveness of sequencing type specimens of coralline algae to link the type bearing name to the many sequences obtained from recent specimens (i.e. Peña *et al.* 2014b, 2015b; Adey *et al.*, 2015; Hernández-Kantun *et al.*, 2015) in the aim to establish a DNA Library of Life (Le Gall *et al.*, 2017). The sequence of the holotype of *Lithophyllum nitorum* allowed us to identify recent collections that expanded its distribution range southwards to the Mediterranean. *Lithophyllum nitorum* is considered a rare species in the British Isles, while our results indicate that it occurs on different substrates in the subtidal, even associated to maerl beds in Brittany and Galicia. According to the literature (Adey & Adey 1973), *L. nitorum* occurs sporadically to the north while increased southwards in Cornwall and the Cantabrian Sea. Together with our confirmed records in the Northwest of Spain (Galicia) and in the Mediterranean, it might represent a temperate *Lithophyllum* species with warm-water affinities; a similar Atlantic-Mediterranean distribution is also reported for *L. incrustans* and *L. hibernicum* (Hernández-Kantun

et al., 2015). Despite the considerable number of publicly available sequences of *Lithophyllum* taxa (more than 275 COI-5P and *psbA* GenBank sequences), none of them matched with *L. nitorum*; the presence of this uncommon, subtidal species with somewhat restricted distribution further supports *Lithophyllum* as a genetically diverse genus in the European coasts (i.e. Pezzolesi *et al.*, 2016). In this regard, if the diversification of the subfamily Lithophylloideae was estimated during the early and middle Miocene (~ 23-12 million years ago, Rösler *et al.*, 2017), the Miocene fossil records of *L. nitorum* in Mediterranean Spain (Braga & Aguirre, 1995) would indicate an early divergence of this species. Finally, our results based on type material confirmed *Lithophyllum nitorum* as a distinct species from other European *Lithophyllum* taxa recently re-assessed (Hernández-Kantun *et al.*, 2015). Numerous additional *Lithophyllum* species from the North East Atlantic and worldwide need to have their type specimens sequenced to correctly apply names.

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