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There is more to maerl than meets the eye: DNA barcoding reveals a new species in Britain, *Lithothamnion erinaceum* sp. nov. (Hapalidiales, Rhodophyta)

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Running title: revealing cryptic diversity within *Lithothamnion*

ABSTRACT

Due to the high plasticity of coralline algae, identification based on morphology alone can be extremely difficult, so studies increasingly use a combination of morphology and genetics in species delimitation. A DNA barcoding study was carried out on maerl-forming coralline algae using the mitochondrial cytochrome oxidase 1 gene, CO1, and the plastid gene, *psb*A, on field specimens from Falmouth and Oban together with herbarium specimens from the Natural History Museum, UK, and the Smithsonian Institution, Washington, USA. Results revealed the presence in the north of Britain of a new species, *Lithothamnion erinaceum* Melbourne & J. Brodie, sp. nov., which was previously misidentified as *Lithothamnion glaciale*. The results also indicated that *Lithothamnion lemoineae*, which had earlier been recorded from Britain, was not present. One of the biggest concerns at present is how organisms will respond to climate change and ocean acidification, and it is imperative that investigations are put on a firm taxonomic basis. Our study has highlighted the importance of using molecular techniques to aid in the elucidation of cryptic diversity.

Key words: Britain, CO1, cryptic diversity, DNA barcoding, *Lithothamnion*, *psb*A, rhodoliths, species delimitation

INTRODUCTION

Non-geniculate coralline algae are a morphologically diverse group with red pigmentation and calcified cell walls (Cabioch & Giraud, 1986), currently placed in various orders of the subclass Corallinophycidae (Le Gall & Saunders, 2007). Unlike geniculate coralline algae (e.g. *Corallina*), they lack non-calcified articulations between calcified segments. Non-geniculate coralline algae exist as crusts or free-living thalli (rhodoliths and maerl). Free-living forms can aggregate to form structurally and functionally complex habitats, maerl beds, that are known to support a high level of biodiversity including commercial species of scallop (Hall-Spencer *et al.*, 2003) and rare and unusual species (Steller *et al.*, 2003; Peña *et al.*, 2014*a*). Maerl beds are found from high to low latitudes and in the low intertidal zones to 150 m depth (Foster, 2001). With their sensitivity to physical disturbances and slow growth rates (average 1 mm yr^{-1}) (Freiwald & Henrich, 1994; Blake & Maggs, 2003; Wilson *et al.*, 2004), maerl beds are seen to be ecologically fragile and a non-renewable resource. The major maerl builders found in Britain, *Lithothamnion corallioides* (P. Crouan & H. Crouan) P. Crouan & H. Crouan and *Phymatolithon calcareum* (Pallas) W.H. Adey & D.L. McKibbin, are protected under the EU Habitats Directive 1992 (Council Directive 92/43/EEC), listed in Annex V (JNCC, 2013), the UK BAP Priority Habitat (Maddock, 2008), and appear on the OSPAR list of threatened and/or declining species and habitats (Region III) (OSPAR, 2008). Maerl beds are also, under the JNCC interpretation of the EC Habitats Directive, a protected habitat within the Annex I category 'sandbanks which are slightly covered by seawater at all times' and Shallow inlets and Bays (JNCC, 2007). Maerl beds already have to withstand human-induced pressures, e.g. dredging (Hall-Spencer & Moore, 2000) and fish farming (Hall-Spencer *et al.*, 2006), that inhibit

their ability to provide complex habitats. With the added stressor of climate change these organisms will be under mounting pressure.

As coralline algae utilize the most soluble polymorph of calcium carbonate, high Mg-calcite, to form their skeletons (Andersson *et al.*, 2008), they are thought to be more susceptible to ocean acidification, the decreasing carbonate saturation state in the ocean caused by increasing levels of anthropogenic CO_2, than other calcifying organisms (Martin *et al.*, 2008). This has led to predictions that calcified macroalgae will be impacted by future global change (Brodie *et al.*, 2014). However, the effect of climate change is species-specific (Ries *et al.*, 2009~~,~~; Noisette *et al.*, 2013) and some species have shown evidence of acclimation to future CO_2 conditions (Ragazzola *et al.*, 2013). It is therefore crucial to put any analysis on a firm taxonomic basis to assess potentially subtle differences in physiology in order to be able to determine how coralline algal habitats will be affected by future climate change.

Some Rhodophyta are extremely difficult to discriminate taxonomically as many specimens lack key identifying features and have highly variable morphologies (Carro *et al.*, 2014). Non-geniculate coralline algae can be particularly difficult to identify using morphological characteristics alone as they acclimate to their local habitats and are known to have convergent morphology (Steneck, 1986). This is due to a wide range of environmental factors that are known to affect maerl and hence the structure of the maerl bed (Wilson *et al.*, 2004; Carro *et al.*, 2014; Dutertre *et al.*, 2015). Since Saunders (2005) pioneered the use of DNA barcoding for red macroalgae, which was soon adopted by Robba *et al.* (2006), there has been an increase in the acceptance of molecular techniques as a tool for species identification. Since then the use of molecular identification has revealed cryptic diversity in all orders of red algae tested (Robba *et*

al., 2006), highlighting the misidentification of species due to misleading morphologies (Hind *et al.*, 2014), and helped to uncover evolutionary history (Hind & Saunders, 2013). Species identification studies of maerl beds in Europe (Carro *et al.*, 2014; Pardo *et al.*, 2014; Hernández-Kantún *et al.*, 2015; Peña *et al*,. 2015*b*), the Caribbean (Peña *et al.*, 2014*b*) and Brazil (Costa *et al.*, 2014) have shown hidden diversity. For example, eight species of maerl-forming taxa, including five *Lithothamnion* species, were identified in maerl beds in Guadeloupe, Caribbean. No sequence matches were found, with only *Lithothamnion* cf. *ruptile* (Foslie) Foslie identified based on a morphological assessment. This, therefore, highlights the previous lack of understanding of the diversity of *Lithothamnion* (Peña *et al.*, 2014*b*). A similar underestimation of diversity has been found in maerl beds elsewhere, such as the Iberian Peninsula. These beds were thought to contain *L. corallioides* and *Phymatolithon calcareum*, but a third species, *P. lusitanicum* V. Peña, was also identified (Peña *et al.*, 2015*a*) gradually replacing both *L. corallioides* and *P. calcareum* southwards.

These studies have shown how DNA barcoding can successfully attribute coralline algae to separate species, regardless of morphology (Carro *et al.*, 2014), and revealed the cryptic diversity within *Lithothamnion*. Studies such as Bittner *et al.* (2011) and Nelson *et al.* (2015) have shown the benefits of systematic and intensive molecular studies in revealing the extent of diversity within Corallinophycidae. Nelson *et al.* (2015) revealed, through a multi-gene phylogenetic study on the New Zealand corallines, a new order called Hapalidiales that now contains the genus *Lithothamnion.* Bittner *et al.* (2011) suggested that as *Lithothamnion* is such a large and important component of the Hapalidiales, delineating its taxon boundaries would be critical in helping to understand the taxonomy and regional diversity of the order.

The genus *Lithothamnion* currently includes 90 taxonomically accepted species (Guiry & Guiry, 2016). It is the largest genus within the subfamily Melobesioideae, with a global distribution (Guiry & Guiry, 2016). *Lithothamnion* species are characterized by their flared epithallial cells, lack of secondary pit connections, presence of lateral cell fusions, long subepithallial initials, uniporate gametangial conceptacles, multiporate tetra/bisporangial conceptacles and a monomerous thallus (Irvine & Chamberlain, 1994). Four species have been reported for the British Isles (Table 1; Irvine & Chamberlain, 1994~~,~~; Brodie *et al.*, 2015): *L. corallioides*, *L. glaciale* Kjellman, *L. lemoineae* Adey and *L. sonderi* Hauck. *L. lemoineae* was described in Adey (1970). The type specimen was collected from Merchant Island, East Penobscot Bay, Maine in 1961 and deposited in the Smithsonian Institution Herbarium (Adey, 1970). The reported presence of *L. lemoineae* in Britain was questioned by Adey & Adey (1973), who considered that *L. lemoineae* was restricted to the north-west Atlantic. However, this view was apparently overturned when in the 1980s two *Lithothamnion* specimens collected from the littoral zone at Whitley Bay, Northumberland (north-east coast of England) were identified as *L. lemoineae* by Yvonne M. Chamberlain and *L. lemoineae* maerl was observed in Orkney by Peter Sansom (Irvine & Chamberlain, 1994).

Differences in the morphological characteristics of these four British species can allow identification (Table 1). *L. lemoineae* generally forms crusts with short, rounded branches roughly 1–2 mm in diameter, is pink-violet to brown in colour when fresh, has large pit connections within the cortical cells and strongly developed cell zonation, and lacks staining bodies (Adey, 1970). Staining bodies (phosphotungstic haematoxylin), were described in crustose corallines by Adey (1966) and thought to be potential storage

proteins (Adey & McKibbin, 1970). *L. glaciale* forms crusts and maerl, with longer, more complex branching that is usually greater than 2 mm in diameter. Specimens are reddish to deep pink in colour when fresh, with small pit connections, weakly developed or no cell zonation and lack staining bodies (Adey, 1970; Irvine & Chamberlain, 1994). *L. sonderi* occurs mainly as flat, thin crusts with small mounds/protuberances (Chamberlain, 1992; Irvine & Chamberlain, 1994). Mature plants have a red glossy texture with white conceptacles, making them easy to identify (Chamberlain, 1992), but young sterile plants are indistinguishable from *L. glaciale* apart from the presence of staining bodies (Irvine & Chamberlain, 1994). *L. corallioides* can occur as crusts but mainly grows in unattached forms with branch thicknesses of < 1 mm, is brownish pink when fresh, and has large primary pit connections within the cortical cells. *L. corallioides* is a warm temperate species mainly found in the south of the UK, whereas *L. glaciale*, *L. sonderi* and *L. lemoineae* are cold temperate more northerly species (Irvine & Chamberlain, 1994). *L. tophiforme* (Esper) Unger is also included here as it is a species found in the arctic and subarctic regions of the north-east Atlantic along with *L. glaciale* (Freiwald & Henrich, 1994; Irvine & Chamberlain, 1994; Adey *et al.*, 2005). *L. tophiforme*, like *L. glaciale*, forms crusts and maerl with longer complex branches > 2 mm thick, is a reddish orange in colour when fresh and contains staining bodies (Adey, 1970). Even though these characteristics provide a first step in distinguishing these species, it can still be difficult to identify them and hence the need for molecular studies on maerl beds (e.g. Carro *et al.*, 2014; Costa *et al.*, 2014; Pardo *et al.*, 2014; Peña *et al.*, 2014*b*).

Using the mitochondrial CO1 and plastid *psb*A gene, an initial DNA barcoding study of the maerl beds in Falmouth and Oban, Britain, as well as historical material

stored in the algal herbarium (BM) at the Natural History Museum, London, UK, was undertaken. This study revealed cryptic diversity within the maerl beds in Scotland and the North of England. The choice of markers in this study followed other molecular identification studies on maerl beds (Carro *et al.*, 2014; Pardo *et al.*, 2014; Peña *et al.*, 2014*b*; Hernández-Kantún *et al.*, 2015). The CO1 marker has been used due to its fast evolution rate and therefore the ability to reveal cryptic diversity and phylogenetic relationships (Hebert *et al.*, 2003*a*, *b*). The plastid *psb*A gene can be seen as a viable alternative for routine DNA barcoding (Carro *et al.*, 2014) and phylogenetic analysis (Bittner *et al.*, 2011). As the *psb*A gene evolves more slowly than the mitochondrial CO1 gene, it is used to recover deep phylogenetic relationships (Yoon *et al.*, 2002) and to resolve intrageneric relationships (Seo *et al.*, 2003~~,~~; Yang & Boo, 2004). The results of the initial barcoding study led to a phylogenetic analysis using historical and contemporary material of *Lithothamnion* around Britain, as well as an assessment of the disparity between molecular and morphological diversity.

METHODS

Crust and maerl specimens from St Mawes Bank, Falmouth, England (mean depth 6 m; July 2014) and Loch Creran, Oban, Scotland (depth 9.6 m; October 2014) were hand collected by scuba divers. Rhodolith, crusts and maerl specimens from the algal herbarium (BM) at the Natural History Museum, London, UK and the type specimen of *L. lemoineae* from the US National Herbarium (USNC), Smithsonian National Museum of Natural History, Washington DC, USA, were included in the analyses. Individual specimens were identified as *Phymatolithon calcareum*, *Lithothamnion corallioides*, *L. glaciale* or *L. lemoineae* based on morphology. In total 35 specimens from Oban, 32 specimens from Falmouth and 33 herbarium specimens from around Britain and Newfoundland, Canada were analysed (Table S1).

DNA extraction, amplification and sequencing

For DNA extraction a sample (1 cm in length), visually free from epiphytes, was taken from the tip of a branch of each specimen. DNA extraction was performed using the QIAamp DNA Micro Kit (Qiagen) following manufacturer's instructions. The type specimen of *L. lemoineae* was sequenced separately to avoid contamination. Two gene fragments were amplified: (1) a standard DNA barcode of 664 bp, the 5' mitochondrial end of the cytochrome oxidase 1 unit (CO1-5P), using primers M13LF3 and M13Rx (Saunders & Moore, 2013); and (2) a 950 bp barcode of the plastid Photosystem II thylakoid membrane protein (*psb*A), using primers psbAF1 and psbAR2 (Yoon *et al.*, 2002).

Each PCR run contained 2.5 µl NH_4 RXN buffer, 1.5 µl $MgCl_2$, 0.5 µl dNTP stock, 0.5 µl Biotaq DNA polymerase (all from Biotaq DNA Polymerase kit, Bioline Ltd., UK), 18 µl PCR quality H_20, 0.1 µl of forward primer, 0.1 µl of reverse primer (each primer had a 10 µM concentration) and 1–2 µl of DNA, totalling 25 µl of the PCR mix. A standard protocol of PCR (initial denaturation 94 °C for 5 min, then 35 cycles of denaturation at 94 °C for 30 s; annealing at 50 °C for 30 s; extension at 72 °C for 1 min; final extension at 72 °C for 5 min and final hold at 10 °C) was used for *psb*A. For CO1 a modified PCR protocol was used (initial denaturation 94 °C for 2 min; then 5 cycles of denaturation at 94 °C for 30 s; annealing at 45 °C for 30 s; extension at 72 °C for 1 min; a further 35 cycles of denaturation at 94 °C for 30 s; annealing at 46.5 °C for 30 s; extension at 72 °C for 1 min; final extension at 72 °C for 7 min and final hold at 10 °C) (Saunders & Moore, 2013).

Amplification success was determined using gel electrophoresis. Samples that had successfully amplified were cleaned using the Illustra GFX PCR DNA purification kit following manufacturer's protocol (GE Healthcare, UK). Dideoxy cycle sequencing was performed on the samples using Big Dye v1.1 (Life technologies, UK) containing 2 ng/100 base pairs of amplicon size and 1 µm primer concentration in 10 µl volume. Amplification was performed using the Techne Thermo cycler (Bibby Scientific) (28 cycles; 10 s at 96 °C, 5 s at 50 °C and 4 min at 60 °C). Excess dye labelled nucleotides from the sequence reaction were removed with ethanol–sodium acetate precipitation and sequenced products were dried, resuspended and run on a 3730 XL capillary DNA analyser (Applied Biosytems). All sequences were submitted to GenBank and all codes can be found in Table S1.

Data analysis

Forward and reverse sequences were aligned and edited using the programmes Seqtrace 0.9.0 (Stucky, 2012) and Bioedit 7.2.5 (Hall, 1999). Bayesian and maximum likelihood optimality criteria were used to analyse phylogenetic relationships (Guindon *et al.*, 2010; Ronquist *et al.*, 2012). Both datasets included sequences from Genbank, related sequences found through BLAST and one outgroup sequence each. *Phymatolithon calcareum* was the chosen outgroup as sequences from type material have already been published. *P. calcareum* is another maerl-forming species in the Hapalidiales, which occupies the same niches as *Lithothamnion* around Britain. It is related yet sufficiently genetically distant to form a suitable outgroup. The CO1 dataset had 14 sequences while the *psb*A dataset had 10 sequences. Jmodeltest v2.1.1 (Guindon *et al.*, 2010; Darriba *et al.*, 2012) was used to select the best fit model by analysing the Akaike information criterion (AIC); the GTR + I model was selected for all datasets.

Bayesian analysis was implemented in MrBayes, version 3.2.3 (Ronquist *et al.*, 2012). Two runs of four chains for 200000 generations were employed for all analyses, with sampling occurring every 50^{th} generation. The mean standard deviation of the split frequencies and the potential scale reduction factor (PSRF) was used to determine the stationarity of the Markov Chain Monte Carlo (MCMC); by 200000 generations all analyses had converged. Consensus trees were constructed after 100000 generations. Maximum likelihood analyses were implemented using PhyML version 3.0 (Guindon *et al.*, 2010) and 100 bootstrap replicates were run to generate bootstrap support statistics (Williamson *et al.*, 2015).

Species delimitation

For species delimitation, the whole sequence dataset was used plus Genbank sequences used in the phylogenetic study, totalling 72 sequences for the CO1 gene and 82 sequences for the *psb*A gene (Table S1). Two different methods were applied, the Automatic Barcode Gap Discovery (ABGD) and the General Mixed Yule Coalescent method (GMYC). The ABGD (Puillandre *et al.*, 2012) aims to statistically find the barcode gap (the gap between intraspecific diversity and interspecific diversity). The method takes a dataset and primarily partitions the dataset based on the gap in the pairwise differences – where the mode of the distribution of intraspecific differences is lower than the mode(s) of interspecific divergence. This method is repeated on the primary partition until no further splitting occurs. The analysis was performed at http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb. All parameters were set to default (P_{min} = 0.001, P_{max} = 0.100, Steps = 10, Number of bins = 20) apart from x, minimum gap width, and the distance. X was set to 1.0, while the Jukes-Cantor (JC69) distance was selected.

Secondly, the GMYC method, developed by Pons *et al.* (2006), aims to delimit species by finding the transition between species level and population level evolutionary processes by analysing differences in branching patterns on a gene tree. A Bayesian phylogenetic analysis was conducted in BEAST v1.5.3. (Drummond *et al.*, 2012) using a GTR + I + G model with a strict clock and using a constant Yule speciation tree prior. Markov chain Monte Carlo (MCMC) analyses were run for 20 million generations for both CO1 and *psb*A. For each run, trees were sampled every 1000th generation. Tracer v.1.5 was used to diagnose the output for convergence, which was when all model parameters for the combined log files exceeded an estimated sample size (ESS) of 200. The GMYC analysis was performed on maximum clade credibility trees calculated with

TreeAnnotator v.1.4.8 and using the SPLITS package for R (http://r-forge.r-project.org/projects/splits/). Both the single threshold and the multiple threshold, where the analysis incorporates a variable transition from coalescent to speciation among lineages (Monaghan *et al.*, 2009), were fitted on a fully dichotomous ultrametric tree.

Morphology

Sporangial conceptacles were observed in histologically prepared specimens that had been decalcified in 1% hydrochloric acid. Longitudinal sections of the decalcified specimens were stained with aniline blue, according to the methods of Brodie & Guiry (1988). Conceptacle measurements were made using ImageJ analysis software (Rasband, 1997–2011).

For cell morphology images, longitudinal sections of the protuberances of specimens were mounted on aluminium stubs and coated with gold. Samples were then imaged using the Hitachi S-3500N scanning electron microscope (SEM). For cell measurements, specimens were scanned using synchrotron-based X-ray tomographic microscopy at the TOMCAT beamline at the Swiss Light Source, Paul Scherrer Institut, Villigen, Switzerland (Stampanoni *et al.*, 2006). For each tomographic scan, 1501 projections over 180 degrees were acquired at 20 keV, 10x objective, field of view of 1.6 x 1.2 mm^2, pixel size 0.65 x 0.65 μm^2. The exposure time ranged between 100 and 200 ms. Projections were rearranged into corrected sonograms, and reconstructed using optimized fast Fourier transformations and gridding procedures (Marone *et al.*, 2010). The final data were exported as TIFF images. Cell measurements were acquired in the Avizo lite V9.0 (VSG) software package. Cell measurements were based on calcified

inorganic material and therefore length and diameter measurements are of the cellular space rather than the whole cell.

RESULTS

Molecular phylogenetic analysis

Analysis of CO1 and *psb*A sequence data of contemporary and historical specimens from Oban and Berwick revealed the presence of both *Lithothamnion glaciale* and an unidentified species of *Lithothamnion*, while specimens from Falmouth were identified as either *L. corallioides* or *Phymatolithon calcareum*. The *psb*A gene of the type material of *L. lemoineae*, collected from Maine, USA, was successfully sequenced and matched with other *L. lemoineae* sequences from Canada. The material initially identified as *L. lemoineae* from Whitley Bay, North Northumberland, Britain, however, did not match the genetic sequences of the type material or Canadian specimens. One specimen (HLE02) matched Genbank sequences of *L. glaciale*, while the other specimen (HLE01) corresponded to *Lithophyllum orbiculatum* and therefore the sequence was not used in the analysis.

Five clades, which represented the five species *Lithothamnion corallioides*, *L. lemoineae*, *L. glaciale*, *Lithothamnion* sp. and *P. calcareum*, were identified in both the CO1 and *psb*A phylogenies (Figs 1, 2). The separation of the warm temperate southern species *L. corallioides* and the colder northern counterparts (*L. glaciale*, *Lithothamnion* sp. and *L. lemoineae*) was supported in both phylogenies (Figs 1, 2). For CO1, the interspecies divergence was 60–67 bp between *L. corallioides* and both *L. lemoineae* and *L.* sp., while it was 57–70 bp difference between *L. corallioides* and *L. glaciale*

(Table 2). For *psb*A, *L. corallioides* differed from *L. glaciale* by 34–40 bp and from *Lithothamnion* sp. and *L. lemoineae* by 33 and 37 bp respectively (Table 3).

In the CO1 phylogeny there was high bootstrap and Bayesian support for the clades representing species (the relation between *L. lemoineae* and *Lithothamnion* sp. was not supported; Fig. 1), whereas in the *psb*A phylogeny high bootstrap and Bayesian support was seen in all clades except for between *L. glaciale* and *L. lemoineae*, where there was only Bayesian support (Fig. 2). The interspecies CO1 divergence within the cold temperate species was 34–45 bp between *L. glaciale* and *Lithothamnion* sp., 50–57 bp between *L. glaciale* and *L. lemoineae* and 50–53 bp between *Lithothamnion* sp. and *L. lemoineae* (Table 2). For *psb*A, the interspecies variation was 12 bp between *L. glaciale* and *Lithothamnion* sp., 12–20 bp between *L. glaciale* and *L. lemoineae* and 13 bp between *Lithothamnion* sp. and *L. lemoineae* (Table 3).

Species delimitation

Results for the ABGD and GMYC (single and multiple) analyses (Table S2, Figs S1–S6) show that for both CO1 and *psb*A, the GMYC model was favoured over the null model for single and multiple thresholds (CO1 (single), $p < 0.001$; CO1 (multiple), $p < 0.001$; *psb*A (single), $p < 0.001$; *psb*A (multiple), $p < 0.001$). For both genes and both thresholds there was significant evidence for the predicted shift in branching rates from interspecific variation to intraspecific variation (Figs S1–S4, Table S2). The multiple threshold for both genes had larger confidence intervals than the single threshold and also over-split the results especially in the *psb*A analysis (Table S2). Using the single threshold, smaller confidence intervals and results comparable to the ABGD analysis and tree topologies were seen within the CO1 data. Therefore, we focussed our

interpretation only on the single threshold results (Table S2). The CO1 gene under the single threshold predicted five clusters, whereas the *psb*A analysis predicted between eleven and twelve clusters with a larger confidence interval. Under the ABGD analysis five groups were delimited for each gene (Table S2, Figs S5–S6). These five groups all included individuals assigned to one of the five species *Phymatolithon calcareum*, *Lithothamnion corallioides*, *L. glaciale*, *L. lemoineae* and *L.* sp. Based on these results coupled with morphological evidence we have therefore described a new species of *Lithothamnion*.

Lithothamnion erinaceum **Melbourne & J. Brodie, sp. nov. (Figs 3, 7–9, 11)**

Diagnosis and description

Initially forming thin smooth crusts over rock, eventually producing small thin protuberances < 3 mm diam.; reddish to deep pink in colour when wet, lighter pink in colour when dry; specimens present all year round; cortical cells 3–11 µm in height, 3–8 µm in diameter; medullary cells 6–11 µm in height, 3–9 µm in diameter; epithallial cells flared; secondary pit connections absent; lateral cell fusions present; tetra/bisporangial conceptacles 140–290 µm diameter, 80–180 µm height with multiporate plates, roof thickness 18–47 µm; pores surrounded by 5–7 cells; CO1/ *psb*A Genbank sequences (KX828508/ KX828452)

Holotype: Loch Creran, Oban, Scotland; coll. Andrew Mogg, 13/10/2014, OB25, BM001150576, deposited in the Natural History Museum, London, UK (BM) (Fig. 3)

Isotype: OB12, BM001150563, deposited in the Natural History Museum, London, UK (BM)

Type locality: Loch Creran, Oban, Scotland, 9.6 m deep in sheltered bay.

Etymology: The name refers to the hedgehog appearance of the holotype and relates to a local name in Scotland of ‘hedgehog stones’.

Habitat: Marine, encrusting on rock, on the west coast found in shallow subtidal areas to 11 m deep (Pardo *et al.*, 2014, see BOLD database), while on the east coast mid-shore to lower intertidal on rocks and in pools.

Distribution: North Atlantic, distributed around Scotland and north England (east coast). Also known in Northern Ireland, Iceland, Norway and British Columbia (Pardo *et al.*, 2014, as *Lithothamnion* sp. 2).

Comparison of Lithothamnion erinaceum sp. nov. *with other cold temperate North Atlantic species*

L. erinaceum sp. nov. exhibits many features of the genus *Lithothamnion* such as flared epithallial cells, primary pit connections, multiporate tetra/bisporangial conceptacles and lateral cell fusions (Figs 7, 8), similar to *L. glaciale* (Fig. 6) (Irvine & Chamberlain, 1994). Apart from molecular data, vegetative characters can also be used to distinguish between different species of *Lithothamnion* (Table 4). When dried, both *L. erinaceum* sp. nov. and *L. glaciale* are a similar deep pink, whereas *L. lemoineae* is characterized by a much lighter pink (Figs 3–5). *L. sonderi* is (mauvy) pink, and covered in white speckles (Chamberlain, 1992). *L. erinaceum* sp. nov. has narrow long branches (typically < 3 mm in diameter), whereas *L. lemoineae* has even narrower yet shorter branches (max 2 mm in diameter). *L. glaciale* branches can be variable in diameter (> 2 mm) (Adey, 1970, Irvine & Chamberlain, 1994) and are typically bulbous (Fig. 4) compared to both *L. erinaceum* sp. nov. (Fig. 3) and *L. lemoineae* (Fig. 5). *L. sonderi* does not form extensive branching like the other cold temperate North Atlantic species of *Lithothamnion*. As branching morphology is related to environmental factors such as depth, light and wave energy (Steller & Foster, 1995), differences seen between environments will be, therefore, manifested within the branching morphology as seen in the Carro *et al.* (2014) study. This means that the branching morphology stated here

may be different in other environments. Hence, caution must be applied in identifying species based on branching morphology in other environments. On the outer surface, the pores of *L. glaciale* are surrounded by 6 cells, whereas *L. sonderi* has a range of between 6 and 8 cells surrounding the pores. *L. erinaceum* sp. nov. also has a range but the number of cells surrounding the pores are between 5 and 7 (Fig. 9). For the reproductive features, *L. glaciale* has buried conceptacles (Irvine & Chamberlain, 1994) (Fig. 10), whereas there is no evidence of buried conceptacles in *L. erinaceum* sp. nov (Fig. 11). The range of asexual conceptacle size is between 80–180 µm in height and 140–290 µm in diameter for *L. erinaceum* sp. nov., which is smaller than the conceptacle sizes for both *L. glaciale* (110–180 µm height, 150–360 µm diameter), and *L. lemoineae* (122–162 µm height, 178–315 µm diameter) (Adey, 1966, Irvine & Chamberlain, 1994). *L. erinaceum* sp. nov has conceptacles larger in height but smaller in diameter than *L. sonderi* (90–140 µm height, 143–422 µm diameter) (Chamberlain, 1992).

DISCUSSION

Despite the long history of the study of maerl around Britain (Irvine & Chamberlain, 1994) our molecular results have uncovered a previously misidentified species of *Lithothamnion* in the maerl beds in the north of Britain. *L. erinaceum* sp. nov. occurs with *L. glaciale* in the maerl beds in Northern Ireland, Scotland, Iceland and Norway, but appears to make a smaller contribution. It was suggested by Pardo *et al.* (2014) that this species, known at the time as *Lithothamnion* sp. 2, along with *L. glaciale* replaces *L. corallioides* and *P. calcareum* as the dominant maerl-forming species at higher latitudes in the North Atlantic. However, they found sequences of this species matching a Pacific Canadian coralline, suggesting that this species may also be found in the North Pacific. Even with the same habitat and similar morphology as *L. glaciale* the significant genetic divergence distinguished *L. erinaceum* sp. nov. as a separate species (Tables 2, 3). This demonstrates the difficulty of relying on morphology alone in the identification of rhodolith and maerl-forming coralline algae and shows that a morphological taxonomy may underestimate the local diversity. The results highlight the hidden diversity within the genus *Lithothamnion* around Britain and the importance of the use of molecular techniques to aid conventional morphological techniques in revealing true diversity, which corroborates earlier findings within the Rhodophyta (Robba *et al.*, 2006~~,~~; Walker *et al.*, 2009~~,~~; Peña *et al.*, 2014*b*).

The results also confirmed the presence of the warm temperate species, *L. corallioides* and *P. calcareum* in the south of Britain and the presence of the cold temperate species *L. glaciale* in the north of Britain. *L. erinaceum* sp. nov., the previously misidentified species, was clearly separated from the other *Lithothamnion* species in the phylogenetic analysis, although the relationship between *L. erinaceum* sp.

nov. and the other cold temperate species (*L. lemoineae* and *L. glaciale*) could not be resolved (Figs 1, 2). This may indicate recent divergence. CO1 is a mitochondrial gene which is expected to evolve faster than the plastid gene *psb*A (Tables 2, 3). The difference in evolution rates of the two genes and our interpretation of a recent divergence would explain why the GMYC model multiple threshold and the GMYC model analysis for the *psb*A gene broke down in delimiting the species and over-split the results (Figs S3, S4). This difference would also explain the ABGD analysis for the *psb*A gene where at a larger prior limit the cold temperate species were collated into one group creating three individual groups instead of five (Fig. S6). These results therefore suggest that more data is required to fully resolve the relationship of these cold temperate species.

Another *Lithothamnion* species requiring further taxonomic study is *L. tophiforme*, an arctic species living in the deeper and colder parts of the subarctic Atlantic (Adey *et al.*, 2005). *L. tophiforme* was first described by Unger in 1858 where he illustrated a specimen from Greenland and stated it was held in the 'hof Naturalien Cabinete'. Unfortunately this specimen has never been found (Adey *et al.*, 2005). The neotype, initially identified as *L. soriferum* Kjellman (Adey *et al.*, 2005), collected from Julianehaab, Greenland, was described in Adey (1970). It is deposited in the Foslie Herbarium, Section of Natural History, Museum of Natural History and Archaeology, Norwegian University of Science and Technology, Trondheim (Woelkerling *et al.*, 2005). There was only one finding of *L. tophiforme* in Britain, in the west of Ireland (Adams, 1908) and the material was unavailable for confirmation (Irvine & Chamberlain, 1994). A small number of molecular analyses have included *L. tophiforme* from Canada, providing SSU (Bailey & Chapman, 1998~~,~~; Adey *et al.*,

2015), cox 2-3 spacer (Hernández-Kantún *et al.*, 2014), rbcL and *psb*A (Adey *et al.*, 2015) genes sequences for this species. However, as the sequences of *L. tophiforme* have not been compared with sequence data of the type material, absolute identification of the specimen must be applied with caution. Ideally type material is sequenced for a confident species assignment. However, as in the case for many crustose corallines, the refusal of destructive sampling, degradation of DNA and lost material type sequencing makes type sequencing not feasible. Hence the second best option is to sequence material that best resembles the original specimen, preferably collected from the type locality. This has also been done for *L. glaciale* and *L. corallioides*, which is recommended here for species assignment.

Even without molecular data, we are confident *L. erinaceum* sp. nov. is not *L. tophiforme* based on morphological differences. *L. tophiforme* is an orange/red colour whereas *L. erinaceum* sp. nov. is more pinkish. *L. tophiforme* has much larger asexual conceptacles, ranging between 222–355 µm in diameter (Adey *et al.*, 2005), than those for *L. erinaceum* sp. nov., which range between 140–290 µm. In addition, there are differences in the geographical distribution between the two species; the waters in Scotland and the north of England can reach temperatures above 12 °C (Inall *et al.*, 2009), which is above the thermal limit of *L. tophiforme* (Adey *et al.*, 2005). As the rest of British coastal waters can reach even higher temperatures, it is unlikely that *L. tophiforme* can survive there and is therefore found further north. However, a study of the rhodolith beds in the Svalbard Archipelago at the Arctic Circle by Teichert *et al.* (2012) found no evidence of *L. tophiforme*. This stresses the need for extensive sampling to further our understanding of the distribution of rhodolith and maerl-forming species in the arctic waters. Specifically, the northern limits of *L. erinaceum* sp. nov.,

the distribution of *L. tophiforme* and whether *L. tophiforme* replaces *L. erinaceum* sp. nov. at higher latitudes all need to be resolved.

We are also confident that *L. erinaceum* sp. nov. is not *L. sonderi* due to clear morphological differences. *L. sonderi* exists mainly as flat reddish crusts with white speckles, sometimes with low mounds/protuberances (Chamberlain, 1992, Irvine & Chamberlain, 1994), whereas *L. erinaceum* sp. nov. exists as more pinkish crusts with prominent branches. The geographic distribution of *L. sonderi* also differs; it is unknown in Iceland and northern Norway as it is unable to withstand the winter temperatures which are below 6–7 ˚C (Adey, 1971). Conversely *L. erinaceum* sp. nov. has been found there and therefore can presumably tolerate these temperatures (Pardo *et al.*, 2014). According to Irvine & Chamberlain (1994) *L. sonderi* does not exist unattached, but can form a crust on dead maerl, whereas *L. erinaceum* sp. nov. grows unattached (Pardo *et al.*, 2014). Attempt to obtain sequence data from *L. sonderi* from around Britain gave variable results: four sequences that did not match each other extremely well, but matched species from the Hapalidiales. However, the species was overgrowing a variety of different crusts. The closest match found in Genbank was for *L. corallioides* at 94% similarity. This suggests that we may be dealing with some new species, in which one may be *L. sonderi*, or even species belonging to different genera. Even though all care was taken in sequencing *L. sonderi*, because the specimens used were crusts overgrowing maerl or growing with a variety of crusts the sequence variation seen could be potentially due to contamination. Therefore, for absolute confirmation of *L. sonderi* fresh material would need to be sequenced and if possible compared with sequence data from the type specimen.

L. lemoineae is a rhodolith/maerl-forming species predominantly found in the North-west Atlantic, from Newfoundland south to the Gulf of Maine. Sequences of historical specimens initially identified as *L. lemoineae*, collected from Britain, did not match those from the type material and were incorrectly identified. Hence, we have disproved the current evidence that *L. lemoineae* is found in Britain (Irvine & Chamberlain, 1994), which is in agreement with Adey (1970) who did not find any evidence of *L. lemoineae* in Europe. This study, therefore, supports the interpretation of *L. lemoineae* being an endemic species in the north-west Atlantic. However, a more extensive study is required to fully rule out the presence of *L. lemoineae* in Britain.

This study has highlighted the need for the use of molecular techniques alongside conventional morphological taxonomy in order to be confident in estimating local species diversity of calcified red algae. Maerl/rhodolith-forming coralline algae are highly plastic organisms known to acclimate to their environments. Large variations in morphology are not only seen within populations but also within individuals. Therefore sole morphological assessment can be extremely difficult. This study shows that molecular taxonomy is an easy method to quickly identify taxa confidently. A full re-evaluation of *Lithothamnion* is needed to understand the distribution and relative abundance of *Lithothamnion* species around Britain.

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Supplementary Information

The following supplementary material is accessible via the Supplementary Content tab on the article's online page at http://xxx

Supplementary Table S1. List of all specimens collected and sequenced, as well as genbank sequences used in the analysis.

Supplementary Table S2. The GMYC (single and multiple threshold) and ABGD analysis of the CO1 and *psb*A genes

Supplementary Figs S1–S4. GMYC analysis, No. of lineages against time. Fig S1; CO1 gene – single threshold. Fig S2; CO1 gene – multiple threshold. Fig S3; *psb*A gene – single threshold and Fig S4; *psb*A gene – multiple threshold. Red line is the transition from interspecies variation to intraspecies variation.

Supplementary Figs S5–S6. ABGD analysis, no of groups against prior intraspecific divergence. Fig S5; CO1 gene and Fig S6; *psb*A gene

Author Contributions

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Tables

Table 1. Species, distribution, colour and the ability to exist as free-living maerl of the four *Lithothamnion* species previously reported to occur in the UK (adapted from Irvine & Chamberlain (1994)).

Species	Biogeography	Distribution	Colour	Free-living?
L. corallioides	warm temperate	Ireland and the south of Britain extending to the Mediterranean	brown/ pink	yes
L. glaciale	cold temperate	replaces *L. corallioides* in the north of the UK and the North East Atlantic	deep pink	yes
L. sonderi	deep water	throughout the North Atlantic	deep red, covered with white speckles	no, but can overgrow dead maerl
L. lemoineae	cold temperate	north-west Atlantic from Canada to Maine, two specimens identified in Northumberland, UK and a personal observation in	pink violet to brown	yes

	Orkney, UK

Table 2. Divergence (bp) for the CO1 gene between *Lithothamnion* species

CO1	*L. glaciale*	*L. corallioides*	*L. erinaceum* sp. nov.	*L. lemoineae*
L. glaciale	0–7			
L. corallioides	57–70	0–6		
L. erinaceum sp. nov.	34–45	60–67	0–2	
L. lemoineae	50–57	60–67	50–53	0–1

Table 3. Divergence (bp) for the *psb*A gene between *Lithothamnion* species

*psb*A	*L. glaciale*	*L. corallioides*	*L. erinaceum* sp. nov.	*L. lemoineae*
L. glaciale	1–4			
L. corallioides	34–40	0		
L. erinaceum sp. nov.	12	33	0	
L. lemoineae	12–20	37	13	0–2

Table 4. Characteristic differences in morphology between cold temperate species of *Lithothamnion* found in Britain, *L. lemoineae*, *L. glaciale*, *L. sonderi* and *L. erinaceum* sp. nov., adapted from Irvine & Chamberlain (1994) and using data from Adey (1966).

	Species			
Character	*L. lemoineae*	*L. glaciale*	*L. erinaceum* sp. nov.*	*L. sonderi*
Colour	pink violet to brown colour	reddish to deep pink with violet tinge	reddish to deep pink in colour	reddish to slightly brownish with white speckles
Thallus appearance	slight gloss	matt	matt	glossy
Branch hardness	hard	hard	hard	n/a
Branch diameter	mainly < 2 mm diameter	variable	mainly < 3 mm diameter	low protuberances/ mature plants warty in appearance
Epithallial cells	flared	mainly flared	evidence of flared cells	flared

Branch anatomy (*L. glaciale* and *L. erinaceum* sp. nov.) / Crustose structure (*L. sonderi* and *L. lemoineae*)				
Cortical cells (l) µm	3.5–12.5	8–12	3–11	2–10
Cortical cells (d) µm	2–7.5	4–8	3–8	5–10
Medullary cells (l) µm	11.5–34.4	4–18	6–16	10–21
Medullary cells (d) µm	3.5–8.5	3–11	3–9	3–9
Tetra/Bisporangial conceptacle (LS)				
Shape		with narrow, non-raised rim, pore plate level	eaised, with slightly arched roof	eaised with flat to slightly arched roof
Roof thickness µm	20–32	5–40	18–47	88–130
Pores	not known	surrounded by 6 cells	Surrounded by 5–7 cells	surrounded by 6–8 cells

Chamber (d) µm	178–315	150–360	140–290	143–442
Chamber (h) µm	122–162	110–180	80–180	90–140
Old conceptacles	Not known	usually become buried	no evidence of buried conceptacles	old conceptacle roofs shed to leave white rimmed craters
Tetra/bisporangia				
Shape	not known	elliptical	not known	long and thin
Length µm	not known	65–96	not known	104–160
Diameter µm	not known	23–47	not known	25–78

1 = length, d = diameter, h = height, n/a = not applicable, * Cell measurements for *L. erinaceum* sp. nov. are based on the cellular spaces within calcified material and therefore not comparable to the other species

Figure Legends

Fig. 1. Maximum Likelihood (ML) phylogram of the CO1 gene. Bootstrap support values for ML/ Bayesian posterior probabilities. Numbers in brackets are number of specimens of each haplotype sequenced in this study that were used in species delimitation analysis.

Fig. 2. Maximum Likelihood (ML) phylogram of the *psb*A gene. Bootstrap support values for ML/ Bayesian posterior probabilities. Numbers in brackets are number of haplotypes sequenced in this study that were used in species delimitation analysis.

Figs 3–5. *Lithothamnion* specimens. Fig. 3. Type specimen of *L. erinaceum* sp. nov. collected from Loch Creran, Oban, UK (BM001150576). Fig. 4. *L. glaciale* collected from Loch Creran, Oban, UK (BM001150562). Fig. 5. *L. lemoineae* collected from Newfoundland, Canada (BM000044415) (Herbarium material). Scale bars: Figs 3–5, 1 cm.

Figs 6–11. Morphological characteristics of *Lithothamnion* species. Fig 6. SEM image of cortical cells of *L. glaciale*. Fig. 7. SEM image of cortical cells of *L. erinaceum* sp. nov. showing flared epithallial cells and primary pit connections. Fig. 8. SEM image of cortical cells of *L. erinaceum* sp. nov. showing lateral cell fusions. Fig. 9. SEM image of a pore of *L. erinaceum* sp. nov. Fig. 10. SEM image showing buried conceptacles in *L. glaciale*. Fig 11. SEM image of *L. erinaceum* sp. nov. with a conceptacle. Legend: Figs. 6–8, black arrow – flared epithallial cells, white arrow- primary pit connections and large black arrow - lateral cell fusions. Scale bars: Figs 6–9, 5 µm; Figs 10–11, 50 µm.