



DNA barcoding allows the accurate assessment of European maerl diversity: a Proof-of-Concept study

BELÉN CARRO*, LUA LOPEZ*, VIVIANA PEÑA, IGNACIO BÁRBARA & RODOLFO BARREIRO

**Both authors contributed equally to this paper*

Grupo BioCost, Facultad de Ciencias, Universidade da Coruña, Campus A Zapateira s/n, 15071-A Coruña, Spain.

E-mail: b.carro@udc.es; lua.lopez@udc.es; vpena@udc.es; barbara@udc.es; rodbar@udc.es

Abstract

Two non-geniculate coralline red algae (*Lithothamnion corallioides* and *Phymatolithon calcareum*) are partially protected under the assumption that they are the main components of maerl beds in Atlantic Europe. However, what we know about the composition of maerl relies mainly on morphology-based identifications that are notoriously difficult due to a lack of diagnostic features, convergence, and widespread phenotypic plasticity. Now, this state of affairs can be improved with new alternatives that, unlike morphology, allow the unambiguous partition of a large number of rhodoliths into species regardless of their size, shape, and condition (fertile or sterile). Here, we report the first DNA barcoding assessment of the relative abundance of maerl-forming algae. The plastidial gene *psbA* was sequenced for 1140 rhodoliths from 15 maerl beds scattered along 2000 km from the British Isles to South Portugal; rhodoliths were randomly collected along linear transects to obtain quantitative estimates of species composition. Most (97%) of our collections belonged to three, rather than two, species that often appeared intermixed along a single transect. *Lithothamnion corallioides* and *P. calcareum* dominated in the British Isles and Brittany (NW France), but they were gradually replaced by *Phymatolithon* sp3 in Galicia (NW Spain) and became extremely rare in Algarve (S Portugal). Morphology (rhodolith size and shape, branch diameter, habit) varied considerably between and within beds but the three species converged to a remarkably similar habit when living in sympatry. Still, *P. calcareum* and *L. corallioides* seemed to perform best in Brittany while *Phymatolithon* sp3 produced the largest rhodoliths with thickest branches in Algarve. Altogether, our study shows that the replacement of species of maerl seen in northern latitudes continues to the south along the coasts of Iberia. It also serves as a proof-of-concept of the benefits of DNA barcoding for ecological and biogeographic research of these taxonomically challenging taxa.

Introduction

In the last decade, DNA barcoding has emerged as an increasingly accepted tool for species identification. Initially devised with an eye on animals (Hebert *et al.* 2003a, b), the approach was rapidly transferred to other taxa with an aim to develop global bio-identification system. In particular, Saunders (2005) pioneered the application of DNA barcoding to red macroalgae and the approach was soon endorsed by Robba *et al.* (2006). Since then, the technique has been widely used for species identification and discovery in the three major seaweeds groups (Heterokontophyta, Rhodophyta, Chlorophyta) (Saunders & McDevit 2012). In fact, it is now acknowledged that molecular techniques have radically altered the way phycological research is conducted, especially in the fields of biodiversity and systematics (Maggs *et al.* 2007). A number of features explain why phycologists have increasingly resorted to molecular tools for routine sample identification: simple anatomy and morphology, remarkable phenotypic plasticity, ample convergence, and incompletely understood life histories (Saunders & McDevit 2012). If these challenging attributes are common in marine macroalgae in general, they are particularly aggravated in the case of the non-geniculate corallines. The latter include important habitat builders. In particular, maerl beds are biogenic habitats built by an accumulation of loose-lying coralline red algae that often harbour high benthic

biodiversity and productivity (Birkett *et al.* 1998, Barbera *et al.* 2003, Peña *et al.* 2014a). These beds are threatened and/or in decline in the European Atlantic and they have been included in the OSPAR Commission (<http://www.ospar.org/>) list of threatened and/or declining habitats (Hall-Spencer *et al.* 2010).

Despite the clear benefits of DNA barcoding, it is only recently that this approach has been applied to non-geniculate corallines. Previous DNA barcode studies on corallines focused mostly on geniculate forms (Robba *et al.* 2006, Walker *et al.* 2009, Hind & Saunders 2013). Nevertheless, Bittner *et al.* (2010, 2011) produced sequences of *psbA* and COI-5P genes for a number of geniculate and non-geniculate corallines, mainly from south Pacific Islands. Also, DNA barcodes were essential to prove that some encrusting gametophytes were conspecific with *Phymatolithon calcareum* (Pallas) W.H. Adey & D.L. McKibbin (1970: 100), a major builder of maerl beds in NE Atlantic (Peña *et al.* 2014b). More recently, as part of a barcoding effort, we used *psbA* and COI-5P sequences in a two-step approach to delimit the various species of maerl in the NE Atlantic finding that many of them have small distribution ranges (Pardo *et al.* submitted). Another interesting outcome of the latter study was that maerl beds might be more diverse than previously thought, at least in some sectors of the European Atlantic. Conventionally, four major species are thought to dominate maerl beds in the OSPAR area: *Lithothamnion glaciale* Kjellman (1883: 123) and *L. tophiiforme* (Esper) Unger (1858: 21) would form maerl in northern latitudes up to the Arctic while *P. calcareum* and *L. corallioides* (P.L.Crouan & H.M.Crouan) P.L.Crouan & H.M.Crouan (1867: 151) would be the major builders of maerl from S Norway to the Mediterranean (Hall-Spencer *et al.* 2010). Instead, our results revealed that beds in Galicia (NW Spain) and Algarve (S Portugal) contain three rather than two abundant species of maerl: *P. calcareum*, *L. corallioides*, and a still undescribed *Phymatolithon* sp3 (Pardo *et al.* submitted). Unfortunately, the true relative incidence of the third species in these beds could not be corroborated with appropriate data as the previous study was designed for assessing species diversity at wide geographic scale without any quantitative purpose.

Here, we fill this gap in our knowledge by showing quantitative estimates of the relative abundance of maerl-forming species in NE Atlantic beds, and their geographical differences, from the British Isles to S Portugal. On the other hand, environmental conditions are known to affect the morphology of maerl. In particular, hydrodynamic conditions and depth have been found to influence both morphology and branch density (Bosellini & Ginsburg 1971, Bosence 1976, 1983, Steller & Foster 1995, Basso 1996, Foster *et al.* 1997, Marrack 1999, Goldberg 2006, Peña & Bárbara 2008). In this regard, and according to the literature, we also recorded several morphological attributes of our specimens to investigate whether the habit, external morphology or branch diameter could serve as a guide for discriminating species in this region (Cabioch 1966, Adey & McKibbin 1970, Adey & Adey 1973, Irvine & Chamberlain 1994, Peña & Bárbara 2004). The accurate species identification of a large number of individuals, most of them sterile, was possible only with the help of molecular tools. Thus, our work is a proof-of-concept example of the benefits of using DNA barcodes as a routine tool when assessing the biodiversity of taxonomically challenging communities.

Material & Methods

Sampling design and sample collection

Samples were collected by SCUBA diving in 15 maerl beds from the British Isles to S Portugal (**TABLE 1, FIGURE 1**). At each site, sampling design consisted of 12–16 samples collected along a linear transect at regular intervals separated by 1–3 m (total transect length 22–39 m). The number of specimens per sample (often >50) varied with maerl density as samples consisted of all the specimens found lying in the vicinity of the transect. Once in the laboratory, all the specimens were air-dried and stored in silica until analysis. Later, each and every specimen from a sample was numbered and 3–6 of them were selected for DNA barcoding identification and morphological analyses by drawing random numbers. In total, 1140 specimens were studied along the study area (32–80 per transect). To account for potential spatial variations in the occurrence of the species of maerl, two rather than one transects were sampled in Milford (480 m apart), Falmouth (220 m), and Algarve (2800 m). In Algarve, the two transects were spatially separated by a shallow rock barrier—10 m deep—that runs *ca.* 11 km parallel to the coast, while in Milford each transect was located on different sides of a fuel jetty.

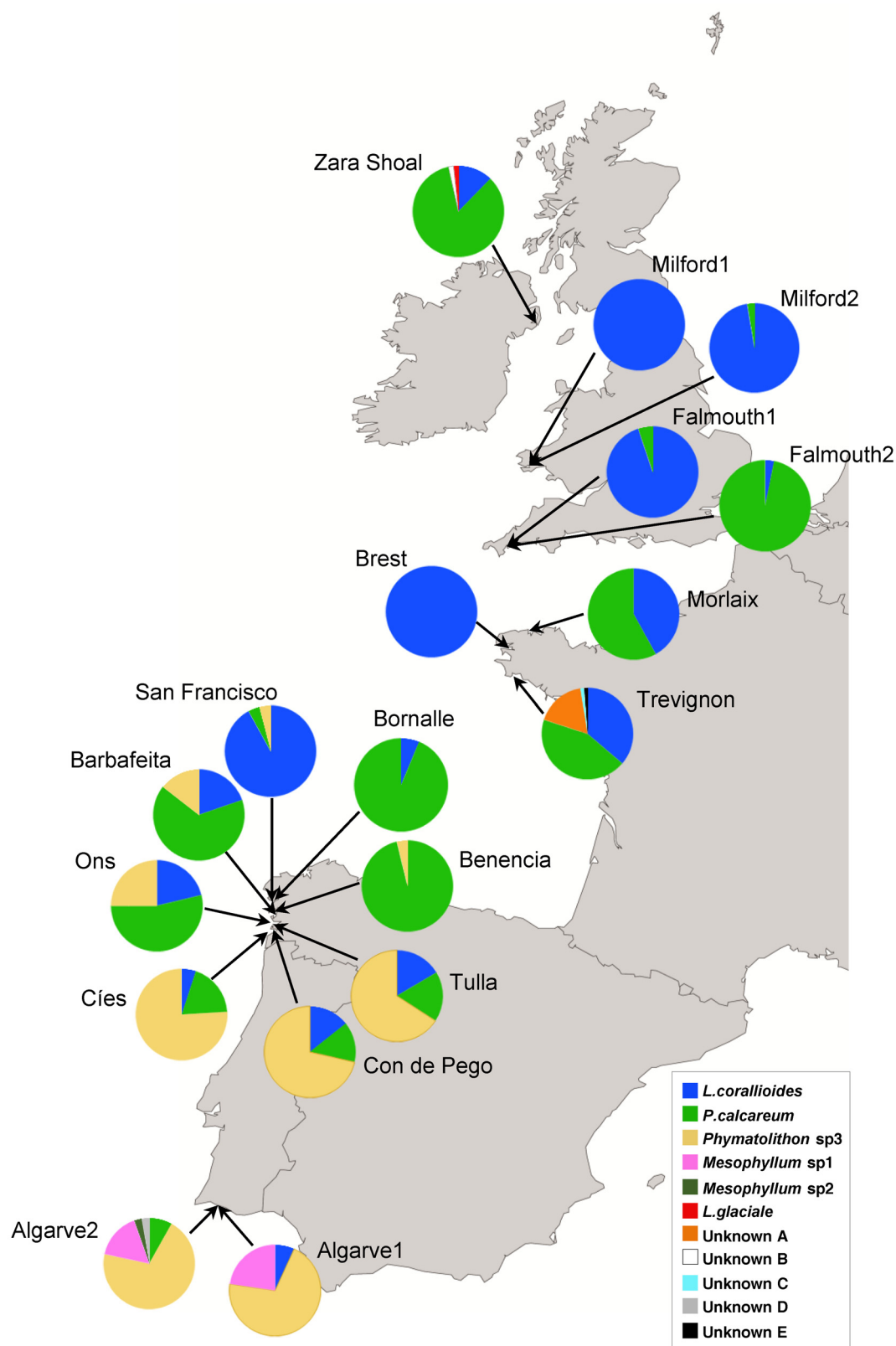


FIGURE 1. Diversity of maerl-forming species in maerl beds of the NE Atlantic. Pie-charts show the relative abundance of 11 maerl species identified with *psbA* sequence data.

DNA extraction, PCR amplification and sequencing

The branched morphology and rugged surface of maerl favors the growth of epiphytes. To ensure that the extracted DNA was uncontaminated with that of other species, each specimen was carefully cleaned under a magnifying glass with the help of an electric mini drill brush before DNA extraction. Extractions were restricted to

the living surface of clean areas obtained by grounding with an electric drill bite. Four plants from Trevignon exhibited two different habits at opposite ends, so two independent samples were collected from each of them. DNA was extracted from 20–100 µL of powdered tissue with the NucleoSpin 96 Tissue kit (Macherey-Nagel, Düren, Germany) following manufacturer's protocols. A fragment of 950 bp of the plastidial gene photosystem II reaction center protein D1 (*psbA*) was PCR amplified with primers *psbA*-F1 and *psbA*-R2 from Yoon *et al.* (2002). In some specimens (see below), a fragment of 664 bp of the 5' end of the mitochondrial gene cytochrome oxidase I (COI-5P, the standard DNA barcode for Rhodophyta) was also amplified with primers GWSLRi and either GWSLF3 or GWSFa from Saunders & Moore (2013). All PCRs were performed in 25 µL containing 2 µL of DNA template, 2.5 µL of 1x PCR buffer, 2.5 mM MgCl₂, 0.192 mM dNTPs, 0.1 µM of each primer, and 1.2 U of AmpliTaq DNA Polymerase (Life Technologies, Carlsbad, USA) in a Biometra TProfessional Basic thermocycler following Saunders & McDevit (2012). After evaluating amplification success by electrophoresis, the excess of primers and nucleotides was removed with Shrimp Alkaline Phosphatase and Exonuclease I enzymes. PCRs products were sequenced using the same primers as for amplification at MacroGen facilities (<http://www.macrogen.com>).

TABLE 1. Sampling localities and number of specimens sequenced per transect. “Samples” is the total number of samples collected at regular intervals along each transect.

Date (2011)	Region	Locality	Transect	Coordinates (UTM)	Depth (m)	Transect length (m)	Samples (n)	Specimens (n)
25/07	British Isles	Zara Shoal	1	30U 0333478E 6028674N	10–10.2	39	14	57
14/07		Milford	1	30U 0356560E 5730106N	5.2–5.3	22	12	37
14/07			2	30U 0356107E 5730270N	5.2–5.6	24	13	38
08/06		Falmouth	1	30U 0355160E 5558779N	3.9–4.7	28	15	39
09/06			2	30U 0355333E 5558919N	5.5–5.7	28	13	32
10/05	Brittany (NW France)	Morlaix	1	30U 0430019E 5395818N	10.4–10.9	30	16	81
16/05		Brest	1	30U 0394752E 5350272N	9.6–10.6	30	16	69
15/05		Trevignon	1	30T 0422545E 5285777N	15–15.2	30	16	80
31/03	Galicia (NW Spain)	Bornalle	1	29T 0498380E 4737354N	11.0	36.4	15	80
31/03		San Francisco	1	29T 0494034E 4733558N	15.0	36.4	15	75
23/06		Benencia	1	29T 0510310E 4716470N	4.0	36.4	15	80
23/06		Barbafeita	1	29T 0508985E 4713006N	6.0	36.4	15	76
07/04		Ons	1	29T 0506975E 4693596N	13.0	36.4	13	80
07/04		Tulla	1	29T 0515581E 4687687N	9.0	36.4	15	79
05/04		Con de Pego	1	29T 0520501E 4678486N	5.0	33.8	14	77
05/04		Cíes	1	29T 0508506E 4673390N	11.0	36.4	15	79
02/03		Algarve (S Portugal)	1	29S 0560723E 4098134N	20.0	35	15	44
23/02			2	29S 0558967E 4100272N	20.0	26	14	37

Species identification

Sequences were edited and aligned using the program Geneious 6.1.7. The *psbA* sequences were compared with those obtained in a previous DNA barcoding study of maerl in NE Atlantic where species were delimited and identified using a combination of molecular (Pons *et al.* 2006, Puillandre *et al.* 2012) and morphological approaches (data available in BOLD—the Barcode of Life Data Systems—at <http://www.boldsystems.org/>). Based on the intraspecific *psbA* sequence variation observed in the BOLD dataset, sequences ≥99.9% identical (*i.e.* ≤1 base difference) to any of those already recorded in the former study were assigned to a species name; otherwise, they were left unassigned. Name assignment was further confirmed with COI-5P sequence data. At least one

specimen of each *psbA* haplotype was sequenced for the COI-5P fragment and these sequences were compared with the COI-5P reference dataset in BOLD. In the case of COI-5P, we used the cut-off value (1%) employed by BOLD for species identification and a sequence was assigned a species name if $\geq 99\%$ similar to any record in the reference dataset, otherwise, it was left unassigned. BLAST (basic local search tool) searches were run in GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) for unassigned sequences to identify the nearest taxonomic group.

Morphological measurements

Size and shape was quantified by measuring the three axes of each specimen. To ensure that the three axes were mutually perpendicular, the longest dimension of the specimen (*a*) and the intermediate axis (*b*) defined the maximum projection plane of the piece while the shortest axis (*c*) was the longest dimension perpendicular to that plane (Sneed & Folk 1958, Foster *et al.* 1997). Rhodolith size was approximated using the volume of an ellipsoid enveloping the thallus following Bosence (1976, 1983). Tests with a random subsample ($N=152$) of our rhodoliths show that this volume is a good predictor ($r^2 = 84.84\%$) of dry weight and a single quantitative relationship fits the three major species of maerl found in our study [Dry weight (g) = $0.45 \times \text{Volume (cm}^3\text{)}$]. The rhodolith shape classes were assessed with ternary diagrams of Sneed & Folk (1958), later applied to rhodoliths (Bosence 1976, 1983, Steller & Foster 1995, Basso *et al.* 2009, Peña & Bárbara 2009) with the help of the Tri-Plot spreadsheet tool developed by Graham & Midgley (2000). Also, branch diameter was measured at the central portion of five branches per specimen to estimate average branch diameter (*D*). All measurements were obtained with vernier calipers to the nearest 0.01 mm. Mean specimen size and *D* values were compared between species using analyses of variance (ANOVA) and after checking that the data met the requirement of homoscedasticity (Levene's test). Since size and *D* changed greatly between locations/transects, ANOVA tests were run on a per transect basis and restricted to cases where two or three species had sample sizes >5 (Zara Shoal, Trevignon, Morlaix, Cies, Con de Pego, Tulla, Ons, Barbafeita).

Results

The *psbA* fragment was successfully amplified and sequenced for 1140 out of 1205 DNA samples (94.6% amplification success). The 1140 sequences included 14 distinct haplotypes with pairwise dissimilarities ranging from 0.1% (1 base difference) to 13.8% (131 base differences). Nine haplotypes comprising 1122 specimens fulfilled the criterion of $\geq 99.9\%$ identity with seven of the maerl species delimited and lodged in BOLD: *P. calcareum* (455 specimens), *L. corallioides* (388), *Phymatolithon* sp3 (253; 2 haplotypes with 1 base difference), *Phymatolithon* sp4 (8), *L. glaciale* (1; Zara Shoal), *Mesophyllum* sp1 (16; 2 haplotypes with 1 base difference), and *Mesophyllum* sp2 (1; Algarve) (**FIGURE 1**). In the two species with more than one haplotype, no geographical difference was observed between haplotypes. Re-checking with COI-5P sequence data reduced the number of name assignments to six as eight rhodoliths initially assigned to *Phymatolithon* sp4 by *psbA* produced COI-5P sequences conspecific with those of *Phymatolithon* sp3. The remaining five *psbA* haplotypes were left unnamed (unknown A–E) and showed pairwise dissimilarities well beyond the values typically observed for conspecifics (7.5–13.8%) that suggests that each one of them possibly belongs to a different species (**FIGURE 1**). These unnamed species were detected in just 18 specimens, most of them (14 specimens from Trevignon, Brittany) were conspecifics (unknown A) while the other four were produced by one single collection each (unknowns C and D from Trevignon, unknown D from Algarve, and unknown B from Zara Shoal). The four rhodoliths from Trevignon that showed different habits in opposite sides of the same piece turned out to be multispecific. In two of them, *L. corallioides* co-existed with the locally common unknown A; the other two combined *P. calcareum* with unknown C and unknown E, respectively. BLAST results placed unknowns A and E within family Corallinaceae, while unknowns B, C and D belonged to family Hapalidiaceae.

The three species that dominated our dataset showed a strong biogeographic pattern with latitude, both in distribution and relative abundance (**FIGURE 1**). Maerl beds in the British Isles and Brittany were mostly built by *P. calcareum* and *L. corallioides*, sometimes in nearly equal abundance (some Breton beds) but more often with a clear dominance of either species. To the south, in the Iberian Peninsula, these two algae shared the formation of maerl beds with another member of genus *Phymatolithon* (*Phymatolithon* sp3). Moreover, the importance of *P. calcareum* and *L. corallioides* decreased the further south the bed was, being gradually replaced by *Phymatolithon*

sp3 and, in Algarve, also *Mesophyllum* sp1. In fact, both *P. calcareum* and *L. corallioides* should be regarded as occasional in our samples from Algarve. This latitudinal replacement could even be perceived at a regional scale in Galicia; thus, the two beds sampled in the northernmost localities (Bornalle and S. Francisco) were dominated by either *P. calcareum* or *L. corallioides* while the two transects collected in the southernmost localities (Con de Pego, Cies) were mostly composed of the meridional *Phymatolithon* sp3, with scarce abundance of *P. calcareum* and *L. corallioides*.

Locally (within transects), there was no clear evidence that the major species of maerl follow a patchy distribution. Instead, in maerl beds with two or more major species, these usually appeared intermingled along transects rather than clumped; moreover, their relative abundance often showed little change from one point of the transect to another (**FIGURE 2**). Nevertheless, some species occasionally showed signs of clumping in some Iberian localities (*L. corallioides* in Tulla, *Phymatolithon* sp3 in Barbafeita) but not in others.

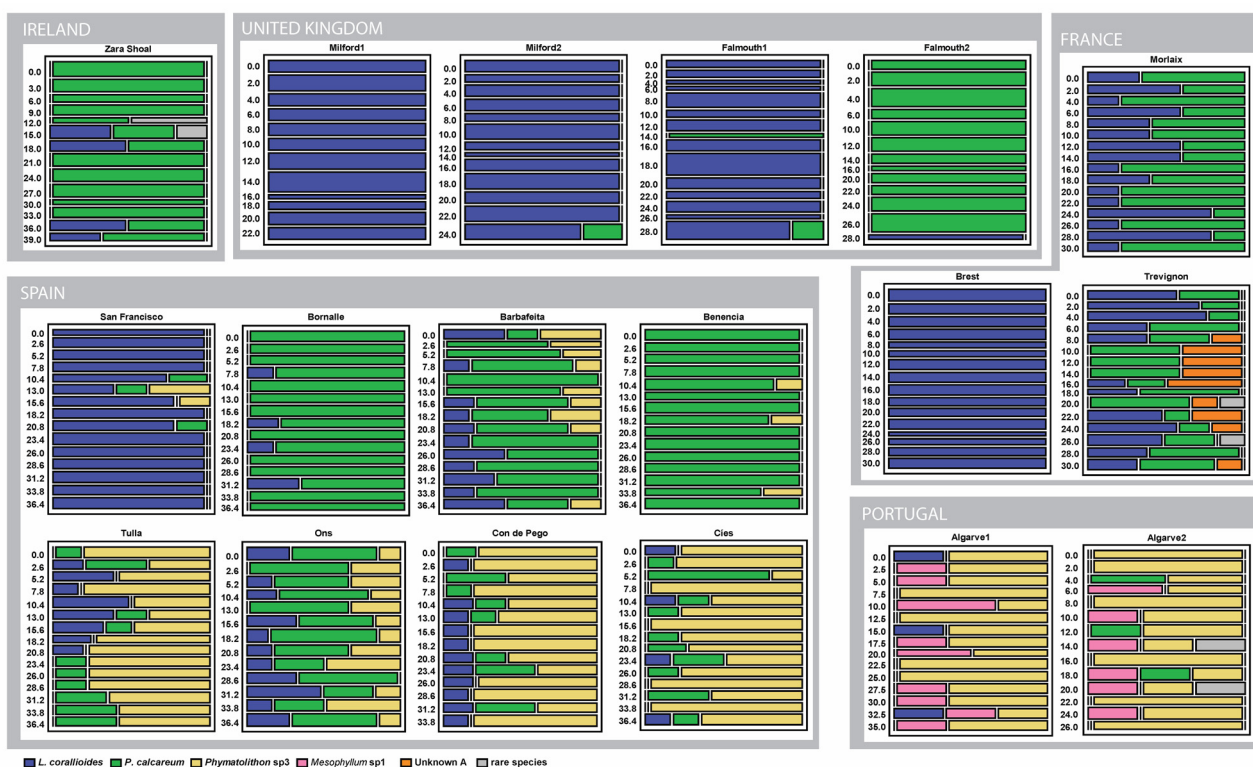


FIGURE 2. Mosaic charts showing the relative abundance of the various species of maerl recorded along 18 sampling transects. Vertical axis is position (in m) along transect; within each chart, width of the bars is proportional to the total number of specimens identified at each position. Thin vertical lines indicate that the species went unrecorded at those positions. For simplicity, species represented by a single record in our dataset are grouped under category “rare species” (i.e. *Mesophyllum* sp2, *L. glaciale* and “Unknown B–E”).

Median rhodolith size was slightly larger in *P. calcareum* (1.47 cm³, *N* = 430) than in the other two species (1.29 cm³ in *L. corallioides*, *N* = 379; 1.26 cm³ in *Phymatolithon* sp3, *N* = 237). Nonetheless, size was very variable, ranging two orders of magnitude in *L. corallioides* (0.16–25.56 cm³) and *P. calcareum* (0.15–58.83 cm³), and one order of magnitude in the Iberian *Phymatolithon* sp3 (0.20–7.88 cm³) (**FIGURE 3A**). In part, size variability was due to differences between locations as median values per transect also changed considerably, ranging from 0.58 cm³ (Milford2) to 11.78 cm³ (Trevignon) in *L. corallioides*, from 0.71 cm³ (Barbafeita) to 15.10 cm³ (Trevignon) in *P. calcareum*, and from 0.63 cm³ (Barbafeita) to 2.07 cm³ (Algarve 2) in *Phymatolithon* sp3. Site conditions, rather than species, seemed the major driver of these changes as the three species often changed their size in the same direction between sites. Thus, Trevignon produced the largest specimens of either *L. corallioides* or *P. calcareum*, followed by Brest (for *L. corallioides*) and Falmouth (for *P. calcareum*); while the three species grew as small rhodoliths in Barbafeita, Ons, and Tulla (Galicia). The largest rhodoliths of the Iberian *Phymatolithon* sp3 were found in our southernmost locations (Algarve, Con de Pego, Cies). ANOVA tests detected significant size differences (*p* < 0.5) between species in some locations (Zara Shoal, Con de Pego) but not in others

(Trevignon, Morlaix, C  s, Tulla, Ons, Barbafeita). In Zara Shoal and Con de Pego, *L. corallioides* specimens were significantly smaller than either *Phymatolithon* sp3 or *P. calcareum*. Branch diameter (*D*) was likewise variable within species, ranging 1.12–5.48 mm in *L. corallioides*, 1.13–4.01 mm in *P. calcareum*, and 1.26–2.76 mm in *Phymatolithon* sp3 (**FIGURE 3B**). Again, most variation was due to differences between sites as *L. corallioides* and *P. calcareum* consistently grew thicker branches in Trevignon and Falmouth2 while *Phymatolithon* sp3 showed the thickest morphology in Algarve. ANOVA tests detected significant ($p = 0.0000$) differences between species in just two Iberian transects (Con de Pego, Barbafeita) where *Phymatolithon* sp3 produced thicker branches than either *P. calcareum* or *L. corallioides*.

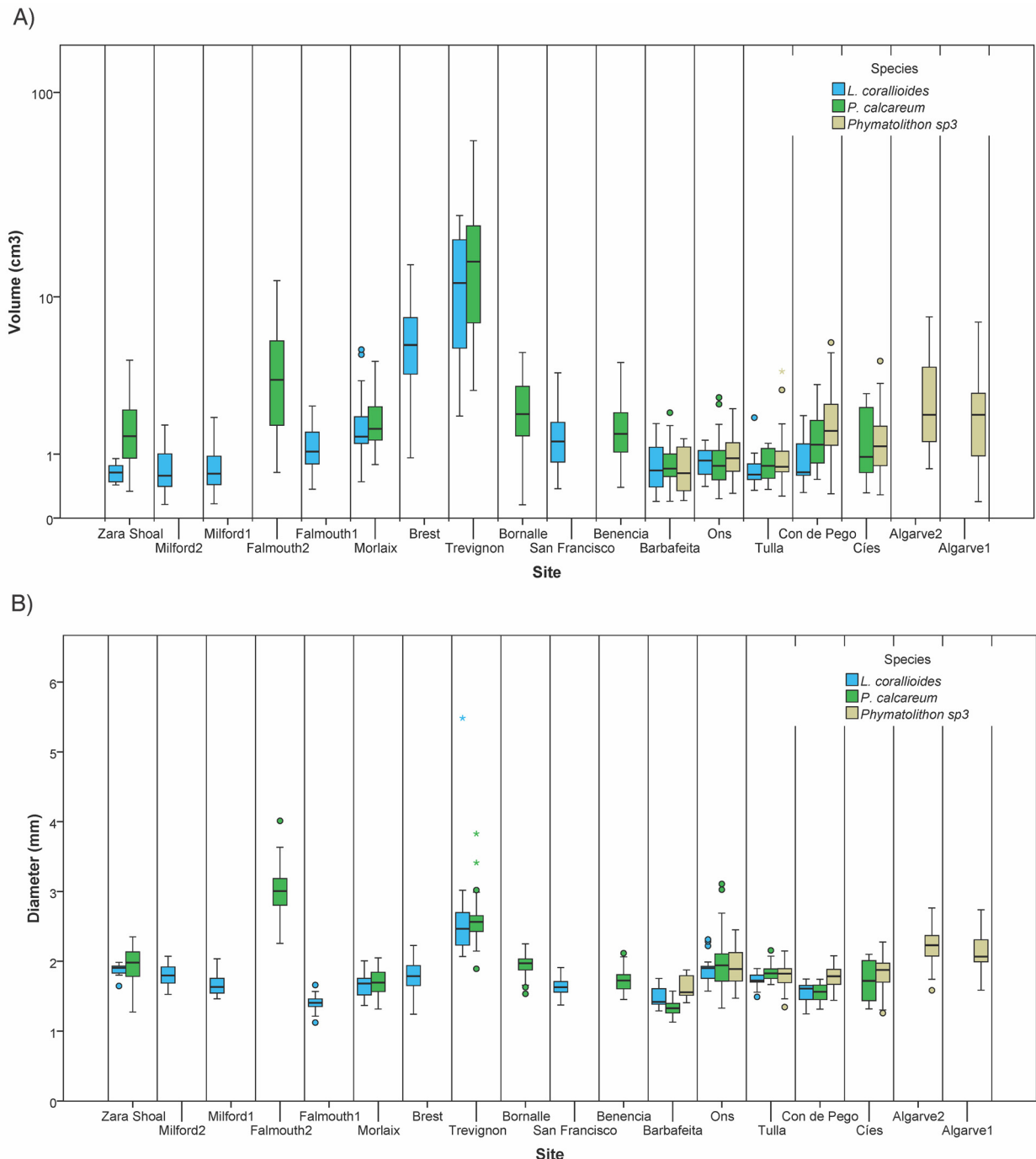


FIGURE 3. Size variability in three major builders of maerl beds in NE Atlantic. Box-and-whiskers plots showing (A) rhodolith volume (cm³) and (B) average branch diameter (mm) estimates for specimens collected in 18 sampling transects covering 15 maerl beds. Each box extends from the lower quartile to the upper quartile, horizontal lines are medians while the lines above and below each box show the smallest and largest data values that were not classified as outliers; outliers (*i.e.* values >1.5 times the interquartile range above or below the limits of the box) are denoted by point symbols. Box-and-whiskers plots show only cases with >5 specimens per transect.

External morphology varied considerably between and within sites but the three species converged to a remarkably similar habit when co-occurring at the same location (**FIGURE 4**). In *P. calcareum* and *L. corallioides*, rhodoliths from Brittany were densely branched resulting in a massive external morphology while more open-branched forms were found in the British Isles and, in particular, Galicia. Despite the remarkable convergence between species, *Phymatolithon* sp3 often displayed a recognizable smooth and matt surface with a greyish pink hue when dry and compressed branches with fan-shaped tips.



FIGURE 4. External morphology of *L. corallioides*, *P. calcareum*, *Phymatolithon* sp3, and its geographical variations along the NE Atlantic. Scale bar = 1 cm.

Rhodolith shape also changed considerably between sites (**FIGURE 5**) as each and every one of the ten shape categories defined by Sneed & Folk (1958) could be found in our dataset. Still, two shape categories were clearly underrepresented; very few rhodoliths were very elongate (bottom right corner in ternary diagrams) or compact (top corner). None of the species had a preferred shape. Instead, shape clearly was site-dependent and the three

species showed similar shape distributions when living in sympatry. An examination of **FIGURE 5** suggests that our 18 transects could be grouped into two broad types of shape distributions. Thus, rhodoliths at eleven transects (Zara Shoal, Milford 1 and 2, Morlaix, San Francisco, Bornalle, Cíes, Tulla, Ons, Algarve 1 and 2) were predominantly discoidal while those at Benencia, Falmouth 1 and 2, Con de Pego, Barbafeita, Brest, and Trevignon were more spheroidal. No obvious relationship between shape distribution and depth was detected.

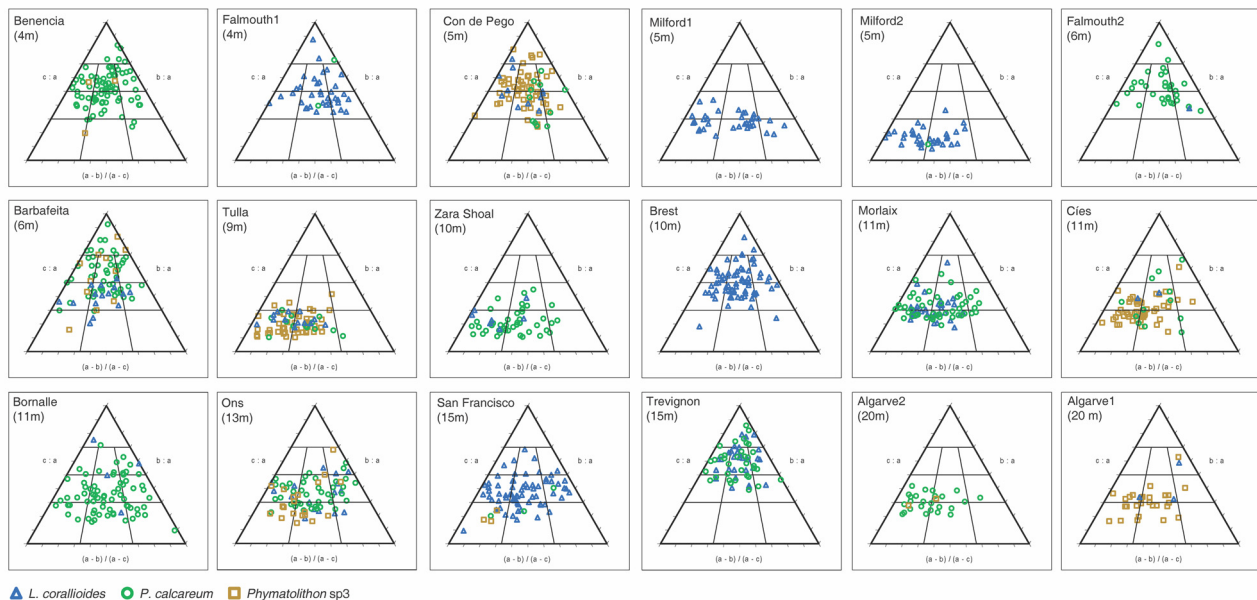


FIGURE 5. Shape variability in three major builders of maerl beds in NE Atlantic. Ternary (Sneed and Folk) diagrams of rhodoliths collected at 18 transects covering 15 maerl beds. Specimens are envisaged as lying in a continuum of forms between spheroidal (top corner), discoidal (bottom left corner), and ellipsoidal (bottom right corner). Data points are estimates of sphericity based on the length of the longest (a), intermediate (b), and shortest (c) orthogonal axes of one rhodolith. Inner lines separate ten finer shape categories defined by Sneed & Folk (1958). Sampling transects arranged according to depth (in m).

Discussion

Our results show that sequence data (*psbA* and/or COI-5P) allow the accurate partition of a large number of rhodoliths into species regardless of their size, shape, and condition (fertile or sterile) providing further support to the benefits of DNA barcoding for red algae in general and corallines in particular (Saunders 2005, Robba *et al.* 2006, Bittner *et al.* 2011, Saunders & McDevit 2012). Moreover, the high amplification success of the *psbA* gene together with increasingly modest costs of external sequencing services suggest that *psbA* sequences could be a feasible alternative for the routine identification of these challenging organisms in ecological and biogeographic research. Rhodolith pre-treatment and DNA extraction was the most time-consuming step in our protocol as we were concerned by DNA contamination from epiphytes. Thus, future attempts would greatly benefit from research finding simpler, faster pre-treatments. Nonetheless, identification with conventional, non-molecular approaches are not necessarily faster, cheaper, or amenable to handle large numbers of samples. The lack of external diagnostic features means that species identification in non-geniculate corallines often requires anatomical examination with scanning electron microscope (SEM) and, even with SEM, species identification may be uncertain if dealing with sterile material. In comparison, sequence data allowed us to discriminate species even in the absence of a proper name and a small number of our samples were assigned to five unnamed entities. The latter was possible thanks to the deep phylogenetic signal observed in our dataset where sequence dissimilarity between species (named or unnamed) was always well beyond the values expected for conspecifics.

Our results show that maerl beds off the coast of Iberian Peninsula contain more species than previously thought; also, they often conceal more diversity than their northernmost counterparts. In the literature, *P. calcareum* and *L. corallioides* were thought to be the major builders of maerl beds in the Iberian Peninsula (Miranda 1934, Seoane-Camba & Campo Sancho 1968, Adey & McKibbin 1970, Peña & Bárbara 2004, 2008, Peña *et al.* 2009, Hall-Spencer *et al.* 2010). Instead, we have found that *Phymatolithon* sp3 is very common in Iberian beds where it

often becomes the primary rhodolith builder (50–75%). Moreover, species composition changes the further south we go and *P. calcareum* and *L. corallioides* go mostly unnoticed in S Portugal where a new, undescribed *Mesophyllum* sp1 is the second component of maerl in terms of abundance. Thus, our results extend the latitudinal replacement of species described for NE Atlantic (Cabioch 1966, Adey & Adey 1973, Irvine & Chamberlain 1994, Birkett *et al.* 1998, Hall-Spencer *et al.* 2010) by showing that the change in species composition continues along the coasts of Iberia. In fact, the Iberian Peninsula undergoes sharp changes as it combines rhodolith communities that are primarily composed of northern species (*P. calcareum*, *L. corallioides*) with others dominated by southern ones (*Phymatolithon* sp3, *Mesophyllum* sp1) in <700 km of coastline. Furthermore, the transition from a northern composition to a more southern one is perceived even at regional scale along Galicia. This fast transition was unexpected since Galician beds are clustered in <70 km and they are all located on apparently similar settings (relatively shallow waters within rias). Thus, our results suggest that, despite their proximity, the environmental differences between the four rias sampled in this study (two beds per ria) must be large enough to explain the change in species composition. A number of pioneer studies have concluded that temperature is the major driver of the replacement of species of maerl with latitude (Adey 1966, Adey & Adey 1973). In this regard, future climate change may result in range shifts due to temperature changes. In the particular case of the region investigated in this study, it could be speculated that *Phymatolithon* sp3 and, perhaps, *Mesophyllum* sp1 might increase their presence in Iberian maerl beds at expense of *P. calcareum* and *L. corallioides* if water temperature increases in the future. Beyond biogeographic, our findings may also have taxonomic implications. Adey & McKibbin (1970) removed *P. calcareum* from genus *Lithothamnion* to *Phymatolithon* after examining abundant material from Ria de Vigo. Now, our results show that the maerl beds from Ria de Vigo (Cíes and Con de Pego in our dataset) are mostly built by *Phymatolithon* sp3 (>70%) while *P. calcareum* is relatively scarce (<20%). Given the notorious morphological similarity between these two species, it cannot be disregarded that some of the descriptions of conceptacle development in *P. calcareum* by Adey & McKibbin (1970) might be based on misidentified plants of *Phymatolithon* sp3. Thus, a thorough description of *Phymatolithon* sp3 seems required to ascertain whether some attribute of its vegetative anatomy or reproductive structures may serve to discriminate this species from *P. calcareum*. On the other hand, previous studies in the European Atlantic regarded branch diameter as a useful external feature to discriminate *P. calcareum* from *L. corallioides*. In this interpretation, branches of *L. corallioides* were usually terete and <1.8 mm in diameter whereas *P. calcareum* had thicker branches (>1.5 mm) with compressed tips (Adey & McKibbin 1970, Adey & Adey 1973, Irvine & Chamberlain 1994, Peña & Bárbara 2004). In comparison, our results show that branch diameter is very variable in both species and it depends more on location than on taxonomy. In fact, we barely detected significant differences in branch diameter between species co-occurring at the same location; and when we did, only *Phymatolithon* sp3 grew thicker branches than the other two, while *P. calcareum* and *L. corallioides* were mostly undistinguishable.

We did not detect any evidence of spatial structuring in the distribution of species within a bed. Instead, the various species of maerl often appear intermixed along a single transect rather than clumped. In comparison, we found that the relative abundance of these species sometimes changes markedly between maerl beds separated by a few km. Thus, in Galicia, the locality San Francisco was primarily composed of *L. corallioides* while the neighbouring Bornalle (5 km upstream within the same ria) was mostly built by an accumulation of *P. calcareum*; likewise, Ons was dominated by *P. calcareum* while Tulla (10 km upstream within the same ria) was primarily composed of *Phymatolithon* sp3. Again, this change in the spatial arrangement of the various species within the same ria suggests the influence of environmental factors. Other authors also found differences in species composition at the scale of a few km. Bosence (1976) found that *L. corallioides* was ubiquitous in Manning Bay, Ireland, whereas *P. calcareum* occurred only in those areas of the bay with quieter waters. We were not able to identify any obvious pattern in our dataset and a denser sampling design will be required to solve this issue.

Rhodolith shape is also thought to vary depending on hydrodynamic conditions, depth, and internal factors not related with water motion (Bosence 1976, Foster *et al.* 1997, Peña & Bárbara 2008, 2009, Basso *et al.* 2009, Rosas-Alquicira *et al.* 2009; but see Steller & Foster 1995). In particular, rhodolith shape has been described to change from discoidal to spheroidal as rhodolith transport increases (Bosellini & Ginsburg 1971, Bosence 1976, Prager & Ginsburg 1989, Bosence 1991). Our results show that different species of maerl converge to a similar habit when living together under identical environmental conditions. In this regard, earlier workers already noted that many species of unattached corallines show the same sequences of morphological variation (Cabioch 1966, Bosence 1976, 1983). However, and unlike previous studies, we did not find an obvious relationship between rhodolith

shape and depth and/or type of coast (open shore, sheltered interior of bays/rias). Thus, we found that spheroidal forms can dominate both deep (*e.g.* Trevignon, 15 m) and shallow sites (*e.g.* Benencia, 4 m), and that discoidal forms can be the primary rhodoliths in either sheltered (*e.g.* Milford) and wave-exposed locations (*e.g.* Algarve). The lack of an obvious pattern in our results could be attributed to the fact that our study combines data from a wide range of rhodolith beds while previous studies often focused on changes within a single or a few beds (Bosellini & Ginsburg 1971, Bosence 1976, Peña & Bárbara 2008, Basso *et al.* 2009, Rosas-Alquicira *et al.* 2009, Gagnon *et al.* 2012, Teichert *et al.* 2012). Nonetheless, further studies including measurements of wave energy and tidal currents at the study sites will be required to assess the actual influence of hydrodynamic energy on rhodolith shape. On the other hand, we observed that largest maerl was linked to deep sites in Brittany (Trevignon, *P. calcareum* and *L. corallioides*) and S Portugal (Algarve2, *Phymatolithon* sp.3). Other authors have reported cases where rhodolith size either increases (Amado-Filho *et al.* 2007, Gagnon *et al.* 2012) or decreases (Steller & Foster 1995, Bahía *et al.* 2010) with depth. However, in our study, the relationship between size and depth is far from clear because other deep sites (San Francisco, Ons) did not follow the pattern and contained small rhodoliths. Trevignon is the only Breton bed where no negative human impacts on maerl structure were detected (Grall & Hall-Spencer 2003). Likewise, a monitoring study conducted in 2006–2009 did not detect any relevant negative impact on the maerl bed of S Portugal (Peña & Bárbara 2013). Alternatively, rhodolith size might be related to the latitudinal replacement of species observed along the study area. It could be speculated that the largest rhodoliths appear at sites closer to the center of the latitudinal range of *P. calcareum* and *L. corallioides* (Trevignon) and *Phymatolithon* sp3 (Algarve), where performance and growth would be better than at other sites.

In conclusion, our study provides the first molecular-assisted, quantitative assessment of the diversity of maerl-forming species in NE Atlantic beds. Species discrimination is notably challenging in non-geniculate corallines where external morphology depends on environment rather than on taxonomy and where different species converge to a remarkably similar habit when living together. In this context, DNA barcoding appears as a powerful tool for the ecological and biogeographic research of maerl forming algae in natural beds. With this tool, we have shown that the latitudinal replacement of species continues along the coasts of Atlantic Iberia where maerl beds are more diverse than previously thought. More importantly, we have been able to obtain estimates of relative abundance that do not depend on rhodolith size, shape, and/or condition.

Acknowledgments

This work was funded by the Ministerio de Ciencia e Innovación of Spain, grant CTM2010-18787 (partially co-funded by FEDER, Fondo Europeo de Desarrollo Regional), and Xunta de Galicia, grant 10MMA103003PR. LL and VP acknowledge fellowships from Universidade da Coruña (Contratos Predoutorais 2012) and Xunta de Galicia (Axudas de apoio á etapa inicial de formación posdoutoral do Plan I2C 2012), respectively. Samples from French Brittany were collected with the support of a European Community-Research Infrastructure Action under the FP7 “Capacities” Specific Programme (ASSEMBLE grant agreement no. 227799). Many colleagues collected material: Cristina Pardo, Daniel Pritchard, Francis Bunker, Jacques Grall, Louise Kregting, Miguel Rodrigues, Pedro Neves, Thomas Wilfried, Verónica García and Yann Fontana.

References

- Adey, W.H. (1966) Distribution of saxicolous crustose corallines in the Northwestern North Atlantic. *Journal of Phycology* 2: 49–54.
<http://dx.doi.org/10.1111/j.1529-8817.1966.tb04593.x>
- Adey, W.H. & Adey, P.J. (1973) Studies on the biosystematics and ecology of epilithic crustose Corallinaceae of the British Isles. *British Phycological Journal* 8: 343–407.
<http://dx.doi.org/10.1080/00071617300650381>
- Adey, W.H. & McKibbin, D.L. (1970) Studies on the maerl species *Phymatolithon calcareum* (Pallas) nov. comb. and *Lithothamnium coralloides* Crouan in the Ría de Vigo. *Botanica Marina* 13: 100–106.
<http://dx.doi.org/10.1515/botm.1970.13.2.100>
- Amado-Filho, G.M., Maneveldt, G.W., Manso, R., Marins-Rosa, B., Pacheco, M. & Guimarães, S. (2007) Structure of rhodolith beds from 4 to 55 meters deep along the southern coast of Espírito Santo State, Brazil. *Ciencias Marinas* 33: 399–410.

- Bahía, R.G., Abrantes, D.P., Brasileiro, P.S., Pereira Filho, G.H. & Amado Filho, G.M. (2010) Rhodolith bed structure along a depth gradient on the northern coast of Bahia State, Brazil. *Brazilian Journal of Oceanography* 58: 232–237.
<http://dx.doi.org/10.1590/s1679-87592010000400007>
- Barbera, C., Bordehore, C., Borg, J.A., Glémarec, M., Grall, J., Hall-Spencer, J.M., Huz, C.D.L., Lanfranco, E., Lastra, M., Moore, P.G., Mora, J., Pita, M.E., Ramos-Esplá, A.A., Rizzo, M., Sánchez-Mata, A., Seva, A., Schembri, P.J. & Valle, C. (2003) Conservation and management of northeast Atlantic and Mediterranean maerl beds. *Aquatic Conservation: Marine and Freshwater Ecosystems* 13: S65–S76.
<http://dx.doi.org/10.1002/aqc.569>
- Basso, D. (1996) Adaptive strategies and convergent morphologies in some Mediterranean coralline algae. *Bollettino della Società Paleontologica Italiana* 3: 1–8.
- Basso, D., Nalin, R. & Campbell, N. (2009) Shallow-water Sporolithon rhodoliths from North Island (New Zealand). *Palaios* 24: 92–103.
- Birkett, D.A., Maggs, C.A. & Dring, M.J. (1998) *Maerl. An overview of dynamics and sensitivity characteristics for conservation management of marine SACs*. Scottish Association for Marine Science (UK Marine SACs Project), Scotland, 116 pp.
- Bittner, L., Halary, S., Payri, C., Cruaud, C., Reviers, B.d., Lopez, P. & Baptiste, E. (2010) Some considerations for analyzing biodiversity using integrative metagenomics and gene networks. *Biology Direct* 5: 47.
<http://dx.doi.org/10.1186/1745-6150-5-47>
- Bittner, L., Payri, C.E., Maneveldt, G.W., Couloux, A., Cruaud, C., de Reviers, B. & Le Gall, L. (2011) Evolutionary history of the Corallinales (Corallinophycidae, Rhodophyta) inferred from nuclear, plastidial and mitochondrial genomes. *Molecular Phylogenetics and Evolution* 61: 697–713.
<http://dx.doi.org/10.1016/j.ympev.2011.07.019>
- Bosellini, A. & Ginsburg, R.N. (1971) Form and internal structure of recent algal nodules (Rhodolites) from Bermuda. *Journal of Geology* 79: 669–682.
<http://dx.doi.org/10.1086/627697>
- Bosence, D.W.J. (1976) Ecological studies on two unattached coralline algae from western Ireland. *Palaeontology* 19: 365–395.
- Bosence, D.W.J. (1983) The Occurrence and Ecology of Recent Rhodoliths — A Review. In: Peryt, T.M. (Ed.) *Coated Grains*. Springer-Verlag, Berlin Heidelberg, pp. 225–242.
- Bosence, D.W.J. (1991) Coralline algae: mineralization, taxonomy and palaeoecology. In: Riding, R. (Ed.) *Calcareous Algae and Stromatolites*. Springer-Verlag, Berlin Heidelberg, pp. 98–113.
http://dx.doi.org/10.1007/978-3-642-52335-9_5
- Cabioch, J. (1966) Contribution à l'étude morphologique, anatomique et systématique de deux Mélobésiées: *Lithothamnium calcareum* (Pallas) Areschoug et *Lithothamnium corallioides* Crouan. *Botanica Marina* 9: 33–53.
<http://dx.doi.org/10.1515/botm.1966.9.1-2.33>
- Crouan, P. & Crouan, H. (1867) *Florule du Finistère*. Klincksieck, F. & Lefournier, J. B. & A., Paris & Brest, 262 pp.
- Foster, M., Riosmena-Rodríguez, R., Steller, D.L. & Woelkerling, W.J. (1997) Living rhodolith beds in the Gulf of California and their implications for paleoenvironmental interpretation. In: Johnson, M.E. & Ledesma-Vázquez, J. (Eds.) *Pliocene Carbonates and Related Facies Flanking the Gulf of California, Baja California, Mexico*. The Geological Society of America, Boulder, Colorado, pp. 127–139.
- Gagnon, P., Matheson, K. & Stapleton, M. (2012) Variation in rhodolith morphology and biogenic potential of newly discovered rhodolith beds in Newfoundland and Labrador (Canada). *Botanica Marina* 55: 85–99.
<http://dx.doi.org/10.1515/bot-2011-0064>
- Goldberg, N. (2006) Age estimates and description of rhodoliths from Esperance Bay, Western Australia. *Journal of the Marine Biological Association of the United Kingdom* 86: 1291–1296.
- Graham, D. & Midgley, N. (2000) Graphical representation of particle shape using triangular diagrams: an Excel spreadsheet method. *Earth Surface Processes and Landforms* 25: 1473–1477.
- Grall, J. & Hall-Spencer, J.M. (2003) Problems facing maerl conservation in Brittany. *Aquatic Conservation: Marine and Freshwater Ecosystems* 13: S55–S64.
<http://dx.doi.org/10.1002/aqc.568>
- Hall-Spencer, J.M., Kelly, J. & Maggs, C.A. (2010) Background document for maerl beds. In: *OSPAR Commission Biodiversity Series*. OSPAR Commission, London, UK, pp. 1–36.
- Hebert, P.D.N., Cywinska, A., Ball, S.L. & DeWaard, J.R. (2003a) Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London Series B-Biological Sciences* 270: 313–321.
<http://dx.doi.org/10.1098/rspb.2002.2218>
- Hebert, P.D.N., Ratnasingham, S. & de Waard, J.R. (2003b) Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London Series B-Biological Sciences* 270: S96–S99.
- Hind, K.R. & Saunders, G.W. (2013) A Molecular Phylogenetic Study of the Tribe Corallineae (Corallinales, Rhodophyta) with an Assessment of Genus-Level Taxonomic Features and Descriptions of Novel Genera. *Journal of Phycology* 49: 103–114.
<http://dx.doi.org/10.1111/jpy.12019>

- Irvine, L.M. & Chamberlain, Y.M. (1994) *Seaweeds of the British Isles: Volume 1: Rhodophyta, Part 2B: Corallinales, Hildenbrandiales*. The Natural History Museum, London, 276 pp.
- Kjellman, F.R. (1883) The algae of the Arctic Sea. A survey of the species, together with an exposition of the general characters and the development of the flora. *Kungliga Svenska Vetenskapsakademiens Handlingar* 20: 1–351.
- Maggs, C.A., Verbruggen, H. & De Clerck, O. (2007) Molecular systematics of red algae: building future structures on firm foundations. In: Brodie, J. & Lewis, J. (eds) *Unravelling the algae: the past, present, and future of algal systematics*. Taylor and Francis, Boca Raton, pp. 103–121.
- Marrack, E. (1999) The relationship between water motion and living rhodolith beds in the Southwestern Gulf of California, Mexico. *Palaos* 14: 159–171.
- Miranda, F. (1934) Materiales para una flora marina de las rías bajas gallegas. *Boletín de la Real Sociedad Española de Historia Natural, Sección Biología* 34: 165–180.
- Pardo, C., López, L., Peña, V., Hernández-Kantún, J., Le Gall, L., Bárbara, I. & Barreiro, R. (submitted) A multilocus species delimitation reveals a striking number of species of coralline algae forming maerl in the OSPAR area. *PLoS ONE*.
- Peña, V. & Bárbara, I. (2004) Diferenciación morfológica y anatómica entre *Lithothamnion corallioides* y *Phymatolithon calcareum* (Corallinales, Rhodophyta) en dos bancos de maerl de la Ría de Arousa (N. O. Península Ibérica). *Anales de Biología* 26: 21–27.
- Peña, V. & Bárbara, I. (2008) Biological importance of an Atlantic European maerl bed off Benencia Island (northwest Iberian Peninsula). *Botanica Marina* 51: 493–505.
<http://dx.doi.org/10.1515/bot.2008.057>
- Peña, V. & Bárbara, I. (2009) Distribution of the Galician maerl beds and their shape classes (Atlantic Iberian Peninsula): proposal of areas in future conservation actions. *Cahiers de Biologie Marine* 50: 353–368.
- Peña, V. & Bárbara, I. (2013) Non-coralline crustose algae associated with maerl beds in Portugal: a reappraisal of their diversity in the Atlantic Iberian beds. *Botanica Marina* 56: 481–493.
<http://dx.doi.org/10.1515/bot-2013-0083>
- Peña, V., Bárbara, I., Berecibar, E. & Santos, R. (2009) Present distribution of maerl beds in the Atlantic Iberian Peninsula. *Museologia Scientifica e Naturalistica volumen speciale IFAA*: 46.
- Peña, V., Bárbara, I., Grall, J., Maggs, C.A. & Hall-Spencer, J.M. (2014a) The diversity of seaweeds on maerl in the NE Atlantic. *Marine Biodiversity* 44(4): 533–551.
- Peña, V., Hernández-Kantún, J., Grall, J., Pardo, C., López, L., Bárbara, I., Le Gall, L. & Barreiro, R. (2014b) Detection of gametophytes in the maerl-forming species *Phymatolithon calcareum* (Melobesioideae, Corallinales) assessed by DNA barcoding. *Cryptogamie, Algologie* 35: 15–25.
<http://dx.doi.org/10.7872/crya.v35.iss1.2014.15>
- Pons, J., Barraclough, T., Gómez-Zurita, J., Cardoso, A., Duran, D., Hazell, S., Kamoun, S., Sumlin, W. & Vogler, A. (2006) Sequence-Based Species Delimitation for the DNA Taxonomy of Undescribed Insects. *Systematic Biology* 55: 595–609.
- Prager, E.J. & Ginsburg, R.N. (1989) Carbonate nodule growth on Florida's outer shelf and its implications for fossil interpretations. *Palaos* 4: 310–317.
- Puillandre, N., Lambert, A., Brouillet, S. & Achaz, G. (2012) ABGD, Automatic Barcode Gap Discovery for primary species delimitation. *Molecular Ecology* 21: 1864–1877.
<http://dx.doi.org/10.1111/j.1365-294x.2011.05239.x>
- Robba, L., Russell, S.J., Barker, G. L. & Brodie, J. (2006) Assessing the use of the mitochondrial *cox1* marker for use in DNA barcoding of red algae (Rhodophyta). *American Journal of Botany* 93: 1101–1108.
<http://dx.doi.org/10.3732/ajb.93.8.1101>
- Rosas-Alquicira, E.F., Riosmena-Rodríguez, R., Couto, R.P. & Neto, A.I. (2009) New additions to the Azorean algal flora, with ecological observations on rhodolith formations. *Cahiers de Biologie Marine* 50: 143–151.
- Saunders, G.W. (2005) Applying DNA barcoding to red macroalgae: a preliminary appraisal holds promise for future applications. *Philosophical Transactions of the Royal Society B-Biological Sciences* 360: 1879–1888.
<http://dx.doi.org/10.1098/rstb.2005.1719>
- Saunders, G.W. & McDevit, D.C. (2012) Methods for DNA barcoding photosynthetic protists emphasizing the macroalgae and diatoms. In: Kress, W.J. & Erickson, D.L. (Eds.) *DNA Barcodes: Methods and Protocols*. Humana Press, Totowa, NJ, pp. 207–222.
- Saunders, G.W. & Moore, T.E. (2013) Refinements for the amplification and sequencing of red algal DNA barcode and RedToL phylogenetic markers: a summary of current primers, profiles and strategies. *Algae* 28: 31–43.
<http://dx.doi.org/10.4490/algae.2013.28.1.031>
- Seoane-Camba, J.A. & Campo Sancho, J. (1968) Resultados de una primera exploración algológica con escafandra autónoma en la Ría de Vigo. *Publicaciones Técnicas de la Junta de Estudios de Pesca* 7: 333–344.
- Sneed, E.D. & Folk, R.L. (1958) Pebbles in the lower Colorado river, Texas. A study in particle morphogenesis. *Journal of Geology* 66: 114–150.
- Steller, D.L. & Foster, M.S. (1995) Environmental factors influencing distribution and morphology of rhodoliths in Bahía Concepción, B.C.S., México. *Journal of Experimental Marine Biology and Ecology* 194: 201–212.
[http://dx.doi.org/10.1016/0022-0981\(95\)00086-0](http://dx.doi.org/10.1016/0022-0981(95)00086-0)
- Teichert, S., Woelkerling, W., Rüggeberg, A., Wisshak, M., Piepenburg, D., Meyerhöfer, M., Form, A., Büdenbender, J. &

- Freiwald, A. (2012) Rhodolith beds (Corallinales, Rhodophyta) and their physical and biological environment at 80°31'N in Nordkappbukta (Nordaustlandet, Svalbard Archipelago, Norway). *Phycologia* 51: 371–390.
<http://dx.doi.org/10.2216/11-76.1>
- Unger, F. (1858) Beiträge zur näheren Kenntniss des Leithakalkes, namentlich der vegetabilischen Einschlüsse und der Bildungsgeschichte desselben. *Denkschriften der Kaiserlichen Akademie der Wissenschaften [Wein], Mathematisch-naturwissenschaftliche Klasse* 14: 13–35.
- Walker, R.H., Brodie, J., Russell, S., Irvine, L.M. & Orfanidis, S. (2009) Biodiversity of Coralline algae in the Northeastern Atlantic including *Corallina caespitosa* sp. nov. (Corallinoideae, Rhodophyta). *Journal of Phycology* 45: 287–297.
<http://dx.doi.org/10.1111/j.1529-8817.2008.00637.x>
- Yoon, H.S., Hackett, J.D. & Bhattacharya, D. (2002) A single origin of the peridinin- and fucoxanthin- containing plastids in dinoflagellates through tertiary endosymbiosis. *PNAS* 99: 11724–11729.
<http://dx.doi.org/10.1073/pnas.172234799>