



## Analysis of the *cox2-3* spacer region for population diversity and taxonomic implications in rhodolith-forming species (Rhodophyta: Corallinales)

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### Abstract

Coralline red algae demonstrate phenotypic plasticity related to environmental factors, rendering their identification difficult. The *cox2-3* spacer is a mitochondrial marker widely used for phylogeographic studies and discrimination between closely related species in red algae; however, *cox2-3* spacer sequence data for coralline algae are still limited. In this study we substantially increase the number of *cox2-3* spacer sequences available for coralline algae, exploring their usefulness for different types of molecular investigations in coralline algae (DNA barcoding and phylogeography), with emphasis on rhodolith-forming species. Specimens from North Atlantic Europe, the Caribbean region and the Gulf of California (Mexico) were sequenced and two datasets were built, one for the subfamily Lithophylloideae and one for the Melobesioideae. Our results suggest the utility of *cox2-3* spacer as barcoding marker for coralline algae with a slight variation in the barcode gap depending in the way gaps in the alignment are treated. Analyses on both datasets found a barcode gap or separation between intra and interspecific divergence (p distance and ABGD analysis) while some inconsistencies were evident when the results were compared with morphology-based classification. Using the *cox2-3* spacer region, the morphospecies *Lithophyllum margaritae* from the Gulf of California revealed the existence of two well-supported clades, with the possibility of respectively five and two additional species; haplotype networks for *Phymatolithon calcareum* and *P. purpureum* revealed similar patterns when Mediterranean and NW Europe specimens were analysed, and *P. calcareum* was shown to consist of a single population in NW Europe. Based on our analyses the marker *cox2-3* spacer has strong potential applications for studies of phylogeography and DNA barcoding in coralline algae after understanding its variation.

**Key words:** Corallinales, *cox2-3* spacer, molecular diversity, cryptic species, intraspecific diversity, interspecific diversity

### Introduction

The *cox2-3* spacer is located in the mitochondrial genome between the genes encoding for the cytochrome oxidase subunit 2 (*cox2*) and 3 (*cox3*). This noncoding region has a higher rate of mutation than the surrounding genes (Zucarello *et al.* 1999, 2006). The *cox2-3* spacer has been accepted as a useful marker for investigation of intraspecific relationships since its first application in red algae (Zucarello *et al.* 1999). Since then, this marker has been used successfully to resolve phylogeographic relationships and/or species delimitation in several red algae, such as *Bostrychia calliptera* (Montagne 1840: 197) Montagne (1842: 661) (Zucarello & West 2002), *Ceramium tenuicorne* (Kützting 1842: 736) Waern (1952: 405) (Gabrielsen *et al.* 2002), *Palmaria palmata* (Linnaeus 1753: 1162) Weber & Mohr (1805: 300) (Provan *et al.* 2005), *Asparagopsis taxiformis* (Delile 1813: 295) Trevisan (1945: 45) (Sherwood 2008), *Batrachospermum* Roth (1797: 36) (e.g., Vis *et al.* 2008, 2010), *Eucheuma* J.Agardh (1847: 16) and *Kappaphycus* Doty (1988: 171) (Conklin *et al.* 2009), *Portieria* Zanardini (1851: 33) (Payo *et al.* 2012), *Fredericqia* Maggs, Le Gall, Mineur, Provan & G.W. Saunders (2013: 284) (Maggs *et al.* 2013), *Gracilaria* Greville (1830: LIV, 121) (Yow *et al.* 2013) and *Bangia* Lyngbye (1819: 82) (Shea *et al.* 2014). Conversely, for *Ahnfeltiopsis pusilla* (Montagne 1834: 287) P.C.Silva & DeCew (1992: 578) this marker did not show any variation among specimens collected over a range of 1200 km, requiring the information of an additional marker (Couceiro *et al.* 2011). In some studies, the *cox2-3* spacer has been used for taxonomic purposes, such as supporting specific delimitation between *Peyssonnelia japonica*

(Segawa 1941: 259) Yoneshige (1985: 200) and *Peyssonnelia boudouresquei* Yoneshigue (1984: 136) (Kato *et al.* 2009). The *cox2-3* spacer has a higher substitution rate than the RuBisCo (Ribulose-1, 5-biphosphate carboxylase oxygenase) spacer region, which is a noncoding region between the large and small subunits encoding for the enzyme (Sherwood 2008). Comparatively, the *cox2-3* spacer has a similar substitution rate to the *cox1* gene, which encode for the Cytochrome Oxidase subunit 1 (*cox1*) and it is used as the barcode marker for many groups of organisms including Rhodophyta (Saunders 2005, Zuccarello *et al.* 2006).

Coralline algae (orders Corallinales and Sporolithales) are calcified members of the Rhodophyta distributed widely on coastal areas as crusts, erected-geniculate or unattached rhodolith in gross morphology (Foster 2001, Nelson 2009). It is known that the gross morphology of coralline algae is strongly affected by phenotypic plasticity related to environmental conditions, which can render their identification difficult (Foster 2001). This is particularly true for certain rhodolith-forming species such as *Lithothamnion corallioides* (P.L.Crouan & H.M.Crouan 1852: no. 242) P.L.Crouan & H.M.Crouan (1867: 151), *Phymatolithon calcareum* (Pallas 1766: 265) W.H.Adey & D.L.McKibbin (1970: 100) and *Lithophyllum margaritae* (Hariot 1895: 167) Heydrich (1901: 530), which have to accommodate to a highly mobile substrate (Irvine & Chamberlain 1994, Riosmena-Rodriguez *et al.* 1999, Foster 2001).

For coralline algae (both Corallinales and Sporolithales), phylogeographic studies using molecular information include only one pioneer study by Vidal *et al.* (2008), who used the *cox2-3* spacer to elucidate phylogeographic patterns in Chilean *Spongites* Kützing (1841: 30) from Chile. Chilean *Spongites* showed 14 haplotypes, but no clear differentiation for haplotypes and geographical regions, which contrasted with a high variation in gross morphology and left questionable the number of species present (Vidal *et al.* 2008). Since Vidal *et al.* (2008) study could not clarify the level of the genetic divergence (intra vs. interspecific diversity) at which the *cox2-3* spacer was informative, a new approach must be taken to study its usefulness to group related taxa and to determine a possible threshold between intra and interspecific diversity.

A barcode gap (threshold) is observed when the genetic divergence of specimens belonging to the same species is smaller (e.g. <1%) than the genetic divergence among related species (e.g. >2%). Recently, with the discovery of an impressive amount of hidden diversity due to morphological similarities, the option of defining hypothetical species using a barcode gap as a first proxy or hypothesis is the starting point for further analysis to be tested. Some other approaches analyse intra and interspecific diversity under a priory-identified species unmasking misidentifications and showing not a clear pattern for the barcoding approach (Meier *et al.* 2006, 2008). In any case, the barcode gap analysis has received contrasting reviews but more analyses are now available to corroborate results and take informed decisions (e.g. ABGD and the barcode gap analysis from BOLD system) (Puillandre *et al.* 2012, BOLD system).

To date, a molecular marker that is useful for corallines to assess species boundaries relative to phylogeography is unknown, or the information is fragmented (Vidal *et al.* 2008). In this study, we expand the dataset of *cox2-3* spacer sequence data available for coralline algae adding numerous new sequences for taxa from Europe, Pacific Mexico and the Caribbean Sea (primarily rhodolith-forming). The variation of the *cox2-3* spacer is examined to evaluate its potential in species delimitation (DNA barcoding) and phylogeography. The results revealed have major repercussions for the taxonomy of *Lithophyllum margaritae* and *Phymatolithon calcareum*, two of the most common rhodolith-forming species.

## Materials and methods

**Taxon selection and sample collection:**—Samples were collected by the first authors and/or collaborators or obtained from herbarium collections (Table 1). Collections were made in NW Europe (northern and Mediterranean France, England, Ireland, Scotland, northern and Mediterranean Spain, Italy), Gulf of California (Mexico) and the Caribbean by SCUBA diving or snorkelling, or at low tide in the lower intertidal. Samples were air-dried for one day and later placed in bags with silica gel for permanent storage. Samples were identified based on gross morphology and anatomical characters using regional taxonomic monographs, Adey & McKibbin (1970), Irvine & Chamberlain (1994), Littler & Littler (2000), Bressan & Babbini (2003), Yabur-Pacheco & Riosmena-Rodriguez (2006) and Riosmena-Rodriguez *et al.* (2012). In some cases, mainly due to a lack of reproductive structures, a definitive identification was not possible. Type specimens for *Phymatolithon calcareum* (sample E160 hereafter) and samples from type localities for *Lithothamnion corallioides* (sample E139, E140 hereafter) and *Lithophyllum margaritae* (sample E110, E116, E108, E57) were obtained and sequenced in order to unambiguously characterize the species concerned. Using Scanning Electron Microscopy (SEM), anatomical features were analysed; fragments of the specimens were mounted

with adhesive film on metal stubs and subsequently coated with gold following standard procedures (Riosmena-Rodriguez *et al.* 1999). Digital images were obtained using a Scanning Electron Microscope Hitachi S-4700 at the National Centre for Biomedical Engineering Science (NCBES), National University of Ireland, Galway. Due to the high molecular diversity observed for *Lithophyllum margaritae*, additional data were generated using anatomical measurements (dimensions of tetrasporangial conceptacles and mature tetrasporangia) using previously obtained SEM images. Specimens from personal collections and recent field trips were deposited in the Phycological Herbarium, National University of Ireland, Galway (GALW), Phycological Herbarium, Universidad Autonoma de Baja California Sur, Mexico (FBCS) and the National Herbarium of the Smithsonian Institution, USA (US).

**DNA extractions, PCR amplification and sequencing:**—DNA was extracted following the modified protocol of the QIAGEN DNeasy Blood & Tissue Kit® (QIAGEN, Crawley, U.K.) by Broom *et al.* (2008). A Techne TC-3000 thermal cycler (Techne, Stone, U.K.) was used for all PCR amplifications. The PCR reactions were carried out using primers designed by Zuccarello *et al.* (1999) and the mix contained 5 µL of Buffer (1x), 2 µL dNTPs (10 mM), 1.5 µL MgCl<sub>2</sub> (1.5 mM), 1 µL of each primer (forward and reverse) (10 pM), 0.3 µL Invitrogen Taq polymerase (1.5x), 38.2 µL HyPure™ Cell Culture Grade Water (Thermo Scientific) and 1 µL DNA template. The PCR protocol consisted of an initial denaturation step at 94 °C for 2 min; 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 1 min and elongation at 72 °C for 1 min; one final cycle at 94 °C for 30 s and a final extension at 72 °C for 3 min. PCR products were visualized and quantified in agarose gels stained with SYBR Safe® DNA stain (Invitrogen, Carlsbad, California) and using HyperLadder II (Bioline, London, U.K.) as reference under UV. The products of successful reactions were purified using the Qiagen MinElute Gel Extraction Kit (Qiagen). PCR products of expected length, yield and purity were sequenced commercially (Macrogen, Seoul, South Korea, or GATC Biotech, Konstanz, Germany) using the same primer pair as in the PCR reaction.

**Molecular genetic analyses:**—Two initial datasets were built with the sequences obtained in the present study: one data set for the subfamily Lithophylloideae and one for the subfamily Melobesioideae. The order Sporolithales has been suggested as the preferable out-group when analysing the Corallinales (e.g. Bittner *et al.* 2011, Kato *et al.* 2011). Nevertheless, samples belonging to the Sporolithales were not found at the time of sampling so, two sequences of *Lithophorella* sp. (classified in the subfamily Mastophoroideae) closely related to the Lithophylloideae and the Melobesioideae were used. *Spongites* sequences produced by Vidal *et al.* (2008) were obtained but they could not be aligned easily, therefore they were excluded from the final analysis. Both datasets were aligned using ClustalW in MEGA version 5 (Tamura *et al.* 2011) with default settings. Analyses to find a barcode gap for the subfamilies Lithophylloideae and Melobesioideae were performed using pairwise distances in Mega 5 treating gaps as 1) pairwise deletion and 2) pairwise inclusion. Each one of the four generated matrices were exported using the option in MEGA 5 to Excel® for Mac 2011, version 14.3.9 Microsoft®. Once the data set was in Excel, distances were transformed to percentage by multiplying the distance values (e.g. value 0.01) by 100 (e.g. result: 1%). After this, percentages were ranked from the lowest to the highest and a graph was built, where y-axis represented percentages of genetic divergence and x-axis represented rank, as used by Puillandre *et al.* (2012). The resulting graph was visually analysed to find an evident threshold identifiable as barcode gap. A second analysis to find primary species hypotheses through barcode gaps included the use of ABGD or Automatic Barcode Gap Discovery for primary species delimitation (Puillandre *et al.* 2012), available in <http://www.abi.nv.jussieu.fr/public/abgd/abgdweb.html> using two of the distance models available, Jukes-Cantor (JC69) and Kimura (K80) TS/TV with Pmin of 0.001, Pmax of 0.1 and a relative gap width of 1.5 for Lithophylloideae and 1 for Melobesioideae (since the option 1.5 for Melobesioideae was rejected by the system due the low divergence in the data set). Two reconstructions were performed for each of the data sets: 1) Neighbour Joining (NJ, a distance analysis) was performed in MEGA version 5 (Tamura *et al.* 2011) using uncorrected p distances (or p distances) and supported by statistical bootstrap of 1000 resamplings, and 2) Maximum Likelihood (ML phylogenetic analysis) was performed using RAxML 1.3 (Mac version: Silvestro & Michalak 2011), with the GTR (General Time Reversible) model with gamma distribution and invariant sites. Bootstrap (BP) analysis with 1000 resampling was used for statistical support. After obtaining some hypotheses of intra- and interspecific genetic divergence in our dataset through barcode gaps (described above), we tested some Hapalidiaceae species for haplotype network reconstruction to elucidate phylogeographic relationships. Haplotype networks were constructed for *Phymatolithon calcareum*, *P. purpureum* (P.L.Crouan & H.M.Crouan 1867: 150) Woelkerling & L.M. Irvine (1986: 71) and *Lithothamnion corallioides*. Each of the three data sets was analysed using the statistical parsimony method of Templeton *et al.* (1992) in the software package TCS version 1.21 (Clement *et al.* 2000) with 95% connection limit.

**TABLE 1.** Specimens analysed in the present study. Herbaria included are GALW (Galway, Ireland), US (Washington D.C., USA) and FBCS (La Paz, Mexico).

Species	Location & collection details	Herbarium	Code	Genbank
<i>Corallinaceae</i>				
<i>Amphiroa rigida</i>	Mahahual, El Faro, Caribbean, México, Subtidal, 30 cm depth, coll. A. Sanchez & J. Hernández, 13 June 2009.	GALW015768	E96	KJ801355
<i>Lithophorella</i> sp.	Sandfly bay, Bocas del Toro, Panama, 2–3 m depth, coll. J. Hernández & N. Robinson, 10 May 2012.	FBCS12904	N34	KJ801362
	Wild cane Cay, Isla Bastimentos, Panama, 3–4 m depth, coll. J. Hernández & N. Robinson, 9 May 2012.	FBCS12905	N42	KJ801363
<i>Lithophyllum byssoides</i>	San Pedro de Cirro, A Coruña, Spain, intertidal, coll. V. Peña & J. Hernández, 24 April 2010.	GALW015732	E72	KJ801352
<i>Lithophyllum margaritae</i> A	Canal San Lorenzo, Isla Espiritu Santo, Mexico, 15 m depth, coll. J. Castillo & J. Hernández, 30 March 2009.	GALW015767	E108, E110, E57	KJ801338, KJ801340, KJ801337
	El Requeson, BCS. Mexico, 10 m depth, coll. J. Lopez, A. Suarez & M. Vergara, 20 June 2009.	GALW015765	E118, E120, E121, E123, E116	KJ801335, KJ801341, KJ801336, KJ801342, KJ801339
<i>Lithophyllum margaritae</i> B	Bahia Magdalena, Pacific, Mexico, 3 m depth, coll. N. Robinson, 2 September 2012.	FBCS12906	E313, E315	KJ801343, KJ801345
	Balandra, La Paz, BCS, Mexico, intertidal, coll. J. Suarez, 23 November 2012.	FBCS12908	E334	KJ801344
	El Requeson, BCS. Mexico, 10 m depth, coll. J. Lopez, A. Suarez & M. Vergara, 20 June 2009.	GALW015761	E52	KJ801348
	Loreto Isla danzante, El Arrollo, BCS, Mexico, 8 m depth, coll. R. Riosmena-Rodriguez, 09 March 2009.	GALW015763	E59, E64	KJ801346, KJ801349
	Punta Blanca, Isla Natividad, Baja California Sur, México, coll. A. Hernández, 10 November 2010.	FBCS12909	E311	KJ801347
<i>Lithophyllum</i> sp.1	El Requeson, BCS. Mexico, 10 m depth, coll. J. Lopez, A. Suarez & M. Vergara, 20 June 2009.	FBCS12910	E104	KJ801350
<i>Lithophyllum</i> sp. 2	Isla Espiritu Santo, Gulf of California, Mexico, coll. J. Martinez, 7 November 2012.	FBCS12911	E337	KJ801351
<i>Lithophyllum</i> sp. 3	Swan Cay, Bocas del Toro, Panama, 2 m depth, coll. J. Hernández & N. Robinson, 16 May 2012.	FBCS12912	N17	KJ801356
	Sandfly bay, Bocas del Toro, Panama, 2–3 m depth, coll. J. Hernández & N. Robinson, 10 May 2012.	FBCS12913	N33	KJ801357
<i>Lithophyllum</i> sp. 4	Brest Bay, France, 4.8 m depth, coll. J. Grall & J. Hernández, 20 July 2010.	GALW015746	E138	KJ801358
<i>Lithophyllum</i> sp. 5	Roscoff, France, tidal pool, coll. J. Hernández, 22 July 2010.	GALW015733	E153	KJ801360
	Roundstone Bay, Ireland, coll. Hanna, 1890.	BM44818	E158	KJ801361
	Cornwall, England, 2 February 2010	US170943	E273	KJ801359
	Kingstown Bay, Co. Galway, Ireland, subtidal, coll. M. Moriarty, 10 November 2009.	GALW015745	E25	KJ801334
<i>Titanoderma</i> sp.	Rocky Reef called “Il Trave”, Portonovo, Ancona, Italy, shallow subtidal, 1 m depth, coll. F. Rindi, 17 July 2012.	US170944	E283	KJ801354
<i>Titanoderma pustulatum</i>	Black Head, Co. Clare, Ireland, intertidal, coll. J. Hernández, 24 May 2011.	US170945	E233	KJ801353
<i>Hapalidiaceae</i>				
<i>Lithothamnion corallioides</i>	Brest Bay, France, 6.2 m depth, coll. J. Grall & J. Hernández, 20 July 2010.	GALW015750	E139	KJ801371

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**TABLE 1.** (Continued)

Species	Location & collection details	Herbarium	Code	Genbank
	Brest Bay, France, 6.2 m depth, coll. <i>J. Grall &amp; J. Hernández, 20 July 2010.</i>	GALW015750	E140, E141, E143	KJ801372 (E140),
	Letter Callow Mill, Kilkieran Bay, Ireland, coll. <i>C.A. Maggs, 13 June 2006.</i>	GALW015751	E166	KJ801375
	Brest Bay, France, 6.2 m depth, coll. <i>J. Grall &amp; J. Hernández, 20 July 2010.</i>	GALW015750	E142, E144	Identical E166
	Carraroe beach, Ireland, 6 m depth, coll. <i>J. Hernández &amp; D. Hanniffy, 9 May 2009.</i>	GALW015749	E115	Identical E166
	Benencia, Galicia, Spain, intertidal, coll. <i>I. Barbara &amp; V. Peña, 22 April 2010.</i>	GALW015748	E70	Identical E166
	Milford Haven, England, subtidal, coll. <i>C.A. Maggs, 01 September 2006.</i>	GALW015747	E129	Identical E166
<i>Lithothamnion glaciale</i>	Loch Sween, Caol, Scotland, subtidal, coll. <i>N. Kamenos, 2 December 2011.</i>	GALW015744	E130	KJ801369
<i>Lithothamnion minervae</i>	Mallorca, Balearic Islands, Spain. Deep sublittoral, lance 121, coll. <i>C. Rodriguez-Prieto, 08 June 2011.</i>	US170946	E236	KJ801379
<i>Lithothamnion muellerii</i>	El Pardito-La Lobera, Isla San Jose, BCS, Mexico, 10 m depth, coll. <i>J. Hernández, 03 December 2008.</i>	GALW015734	E63	KJ801387
<i>Lithothamnion tophiforme</i>	Gull Roce Louber, Bonne Bay, Newfoundland, Canada, 17 m depth, coll. <i>unknown, 10 November 2001.</i>	US170947	E296	KJ801383
<i>Lithothamnion</i> sp. 1	Canal San Lorenzo, Isla Espiritu Santo, Mexico, 15 m depth, coll. <i>J. Castillo &amp; J. Hernández, 30 March 2009.</i>	FBCS12914	E109, E107	KJ801367, KJ801366
	San Esteban, La Paz, Gulf of California, Mexico, intertidal, coll. <i>R. Riosmena-Rodriguez, October 2011.</i>	FBCS12916	E329	KJ801385
<i>Lithothamnion</i> sp. 2	Swan Cay, Bocas del Toro, Panama, 2 m depth, coll. <i>J. Hernández &amp; N. Robinson, 16 May 2012.</i>	FBCS12917	N8	KJ801365
	Isla San Pedro Martir, Mexico, 17 m depth, coll. <i>J. Hernández &amp; A. Suarez Castillo, 12 May 2009.</i>	FBCS12922	E203	KJ801377
<i>Lithothamnion</i> sp. 3	El Requeson, BCS. Mexico, 10 m depth, coll. <i>J. Lopez, A. Suarez and M. Vergara, 20 June 2009.</i>	GALW015738	E124	KJ801368
<i>Lithothamnion</i> sp. 4	Ascension Island, Atlantic, subtidal, coll. <i>J. Hall-Spencer, 2006.</i>	FBCS12919	E156	KJ801373
	Swan Cay, Bocas del Toro, Panama, 2 m depth, coll. <i>J. Hernández &amp; N. Robinson, 16 May 2012.</i>	FBCS12920	N1	KJ801364
<i>Lithothamnion</i> sp. 5	San Esteban, La Paz, Gulf of California, Mexico, intertidal, coll. <i>R. Riosmena-Rodriguez, October 2011.</i>	FBCS12921	E328	KJ801384
<i>Melobesia membranacea</i>	Finavarra, Co. Galway, Ireland, intertidal, coll. <i>J. Hernández, 15 May 2011.</i>	US170948	E232	KJ801378
<i>Mesophyllum engelhartii</i>	Canal San Lorenzo, Isla Espiritu Santo, Mexico, 15 m depth, coll. <i>J. Castillo &amp; J. Hernández, 30 March 2009.</i>	GALW015773	E67	KJ801388
<i>Mesophyllum</i> sp.	Marseilles, France, Code QUB Med 6, coll. <i>C.A. Maggs, 18 November 2004.</i>	US170949	E135	KJ801370
<i>Phymatolithon calcareum</i>	Neotype and Isoleotypes <i>Phymatolithon calcareum</i> , Falmouth, England, coll. <i>W.F. Farnham, 11 December 1983.</i>	BM 000712373	E160, E161, E162, E163	KJ801374 (E160)
	Zarg Shoal, Strangford, Lough. Northern Ireland, 10 m depth, coll. <i>S. Vize, 2003.</i>	GALW015755	E164, E165	Identical E160
	Gleesk, Co. Kerry, Ireland, subtidal, coll. <i>C.A. Maggs, 25 June 2006.</i>	GALW015756	E168, E169, E170	Identical E160

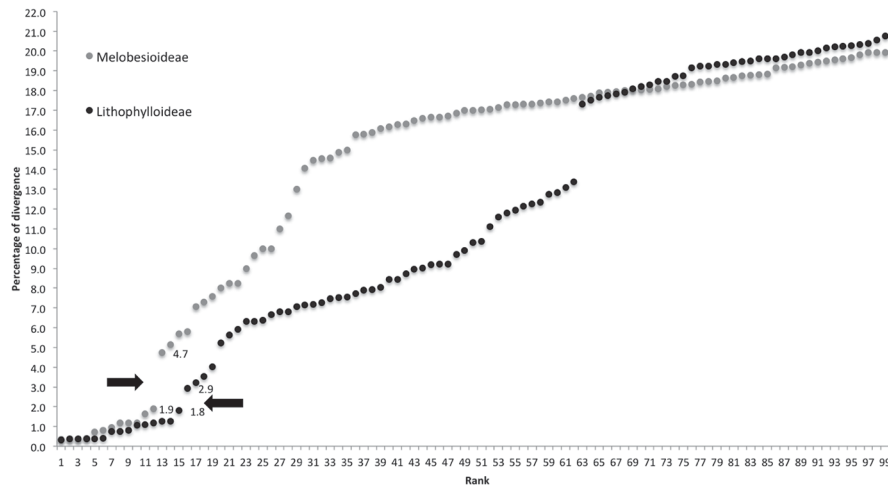
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**TABLE 1.** (Continued)

Species	Location & collection details	Herbarium	Code	Genbank
	Clew Bay, Ireland, subtidal, coll. C.A. Maggs, 06 July 2006.	GALW015757	E173, E174	Identical E160
	Kilkieran Bay, Kilbrickan, Ireland, subtidal, coll. C.A. Maggs, 15 June 2006.	GALW015758	E178	Identical E160
	Aran Islands, CV10-04-05, Ireland, coll. S. Joshi, 15 February 2010.	GALW015759	E180	Identical E160
	Mannin Bay, Co. Galway, Ireland, 2–3 m depth, coll. J. Hernández & N. Nolan, 10 November 2011.	GALW015760	E183, E184	Identical E160
	Benencia, Galicia, Spain, intertidal, coll. I. Barbara & V. Peña, 22 April 2010.	GALW015752	E77, E78, E79, E80, E88	Identical E160
	Old Harry Dorset, England, 17 m depth, coll. L. Baldock, 23 July 2006.	GALW015753	E128	Identical E160
	Falmouth, England, 18 m depth, coll. J. Hall-Spencer, 2 June 2010.	GALW015754	E154, E155	Identical E160
	Mallorca lance 127, Balearic Islands, Spain, 54 m depth, coll. C. Rodriguez-Prieto, 10 June 2011.	US170950	E246, E266	KJ801381 (E246)
	Minorca lance 139, Balearic Islands, Spain, 59 m depth, coll. C. Rodriguez-Prieto, 13 June 2011.	US170951	E267	Identical E246
<i>Phymatolithon purpureum</i>	Mukinish, Ireland, intertidal, coll. J. Hernández, 4 November 2009.	GALW015740	E20, E37, E38,	KJ801376 (E20)
	Carraroe beach, Ireland, 6 m depth, coll. J. Hernández and D. Hanniffy, 9 May 2009.	GALW015741	E113	Identical E20
	Mallorca lance 123, Balearic Islands, Spain, 64 m depth, coll. C. Rodriguez-Prieto, 9 June 2011.	US170952	E242	KJ801389
	Mallorca lance 127, Balearic Islands, Spain, 54 m depth, coll. C. Rodriguez-Prieto, 10 June 2011.	US170953	E245	KJ801380
	Minorca lance 139, Balearic Islands, Spain, 59 m depth, coll. C. Rodriguez-Prieto, 13 June 2011.	US170954	E269, E270	E269 identical E245, KJ801382 (E270)
<i>Phymatolithon</i> sp.	Isla San Pedro Martir, Mexico, 17 m depth, coll. J. Hernández & A. Suarez Castillo, 12 May 2009.	FBCS12922	E203	KJ801377
Unidentified E58	Canal San Lorenzo, Isla Espiritu Santo, Mexico, 15 m depth, coll. J. Castillo & J. Hernández, 30 March 2009.	GALW015736	E58	KJ801386

## Results

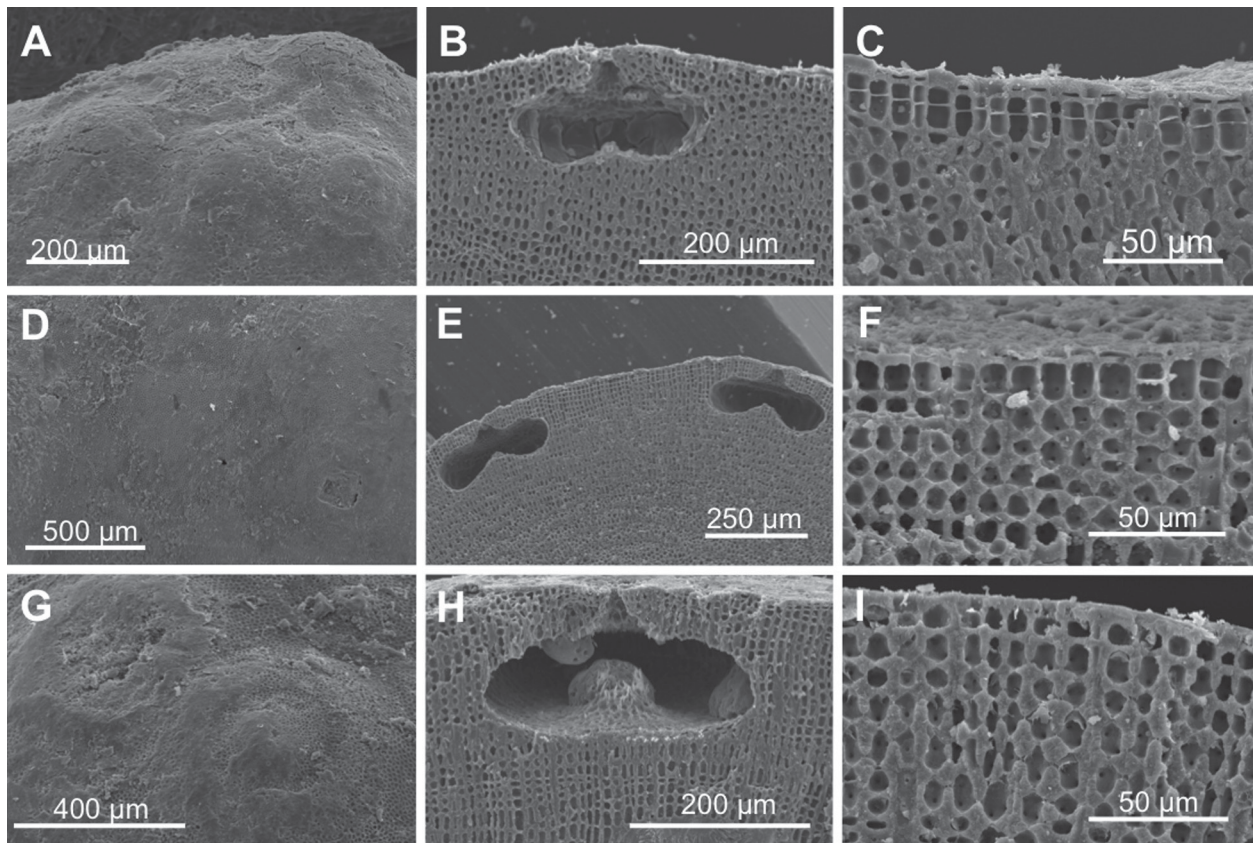
A total of 91 sequences were generated in this study. Removing identical sequences for the analysis of the two families resulted in 30 for the Lithophylloideae and 28 for the Melobesioideae (Supplementary material Document 1 alignment). Within the Lithophylloideae, sequences ranged from 249–328 base pairs (bp) and for the Melobesioideae from 222–331 bp (Supplementary material Document 2 alignment). Alignment gaps (e.g. TT-TT vs. TTATT) were introduced by the program MEGA when markedly different sequences (belonging to different genera) were aligned, whereas highly similar sequences could be easily aligned without gaps. Pairwise distance analyses revealed a threshold or barcode break between 1.9–4.7% when taking into account alignment gaps for the Melobesioideae (Fig. 1). The value was reduced when alignment gaps were excluded (Table 2, supplementary material Fig. 1). In the Lithophylloideae, a threshold or barcode break was found between 1.8–2.9% when taking into account alignment gaps (Fig. 1). This increased when the alignment gaps were excluded (Table 2, supplementary material Fig. 1).



**FIGURE 1.** Pairwise distance for each pair analysed in Melobesioideae and Lithophylloideae including alignment gaps in the dataset. Y-axis represents percentage of divergence while X-axis represents the rank detected in the data set. An arrow marks the threshold found and the divergence values (%).



**FIGURE 2.** Neighbour Joining analysis of the *cox2-3* spacer for members of the subfamily Lithophylloideae. Bp values are reported at nodes in the form Neighbor-Joining (NJ)/Maximum Likelihood (ML), respectively; nodes marked with ‘-’ are not supported (>50%). Numbers in circles represent haplotypes found in the present study and are used in the text as reference. Black bars demarcate the same hypotheses suggested by the p distance threshold and ABGD analysis using Jukes-Cantor (JC69) and Kimura (K80 TS/TV) models. Sand bar demarcates the morphospecies based on anatomical examination and identification.



**FIGURE 3.** Comparative anatomy of specimens referred as *Lithophyllum margaritae* group A and B. A. Specimen E53, identical sequence with E108 (haplotype 2 in Fig. 2) showing the surface view of tetrasporangial conceptacles. B. Specimen E53 transverse view of tetrasporangial conceptacle. C. Specimen E53, detail of transverse section showing meristem cells. D. Specimen E315 (haplotype 10 in Fig. 2) showing the surface view of tetrasporangial conceptacles. E. Specimen E315, transverse view of tetrasporangial conceptacle. F. Specimen E315, detail of transverse section showing meristem cells. G. Specimen E311 (haplotype 13 in Fig. 2) showing the surface view of tetrasporangial conceptacles. H. Specimen E311 transverse view of tetrasporangial conceptacle. I. Specimen E311, detail of transverse section showing meristem cells. Anatomical variation was found only in reproductive structures (conceptacle dimensions and tetraspores packs) while vegetative features were alike (e.g. meristem).

### *Lithophylloideae*

The NJ analysis for the subfamily Lithophylloideae recovered similar topologies and bootstrap support as the ML, for which only the earliest is represented, including both bootstrap values in the way of NJ/ML hereafter. In this reconstruction, the taxa represented belonged to three genera, *Lithophyllum* Philippi (1837: 387), *Amphiroa* Lamouroux (1812: 185) and *Titanoderma* Nageli in Nageli & Cramer (1858: 532). The species of *Titanoderma* formed a clade with moderate/high support (90/73, Fig. 2). Members of the genus *Lithophyllum*, as defined on morphology basis, did not cluster (Fig. 2); however, several sequences grouped for certain morphospecies as in the case of *Lithophyllum* sp. 4 (traditionally called *L. fasciculatum* Lamarck (1816: 203) Foslie (1898: 10) and *Lithophyllum* sp. 5 (a cluster of morphospecies, *L. incrustans* Philippi (1837: 388) and *L. hibernicum* Foslie (1906: 24)). The analysis for the subfamily Lithophylloideae recovered six moderately to highly supported internal nodes out of 13, including values 100/68 (samples E57, E108, E116, E110, E120, E123, E118, E121), 100/86 (samples E313, E315, E334, E59, E311, E52, E64), 99/96 (samples E104, E337), 90/73 (samples E233, E283), 100/99 (samples N17, N33) and 99/85 (samples E138, E25, E153, E273, E158). Terminal nodes for the above clades were supported by lower values of bootstrap (e.g. 65/71 samples E57, E108); in only one case a terminal node was highly supported 100/88 (samples E118, 121) (Fig. 2). Furthermore, specimens identified as *Lithophyllum margaritae* were subdivided in two separated clades (A and B, Fig. 2) with high bootstrap support (100/68 and 100/86 respectively, Fig. 2) and a divergences ranging from 17.3 to 23.2% (Supplementary material Table 1). Within *L. margaritae* A distance values among taxa of different branches ranged from 0.7% (Haplotype 1 vs. 2) to 13.1% (Haplotype 4 vs. 7) (Supplementary material Table 1). Distance values among branches for *L. margaritae* B ranged from 0.3% (Haplotype 9 vs. 10) to 9.7% (Haplotype 11 vs. 14) (Supplementary material Table 1). Using the values of the barcode break found (1.8–2.9%), we recovered intra- and interspecific hypotheses graphically shown in Fig. 2. ABGD analysis using Jukes-Cantor (JC69) and Kimura (K80



TS/TV) models recovered the same hypotheses. Three incongruences were observed when comparing morpho-species and the results from the two models and the barcode gap by p distance: *Lithophyllum margaritae* A was subdivided in five hypothetical species while *Lithophyllum margaritae* B was subdivided in two hypothetical species (Fig. 2); conversely, *Lithophyllum* sp. 1 and sp. 2 were included in the same species. The morphospecies *Lithophyllum margaritae* presented different sizes of the tetrasporangial conceptacles and dimensions of the mature packs of tetraspores for each of the groups observed as A and B (Table 2, Fig. 3, no statistical test was possible since only three useful specimens were available). Additional anatomical differences were not found among members of *L. margaritae* B (Table 3, Fig. 3D–I), and reproductive structures were not observed in distant haplotypes for *L. margaritae* A.

**TABLE 2.** Percentage values of Maximum and Minimum values for barcode gaps and their absolute value or window for *cox2-3* spacer region.

	Melobesioideae (%)	Lithophylloideae (%)
Max. and min. values for the barcode break (including gaps in the alignment)	1.9–4.7	1.8–2.9
Absolute value of the barcode gap (including gaps in the alignment)	2.8	1.1
Max. and min. values for the barcode break (excluding gaps in the alignment)	1.9–3.8	2.3–3.4
Absolute value of the barcode gap (excluding gaps in the alignment)	1.9	1.1

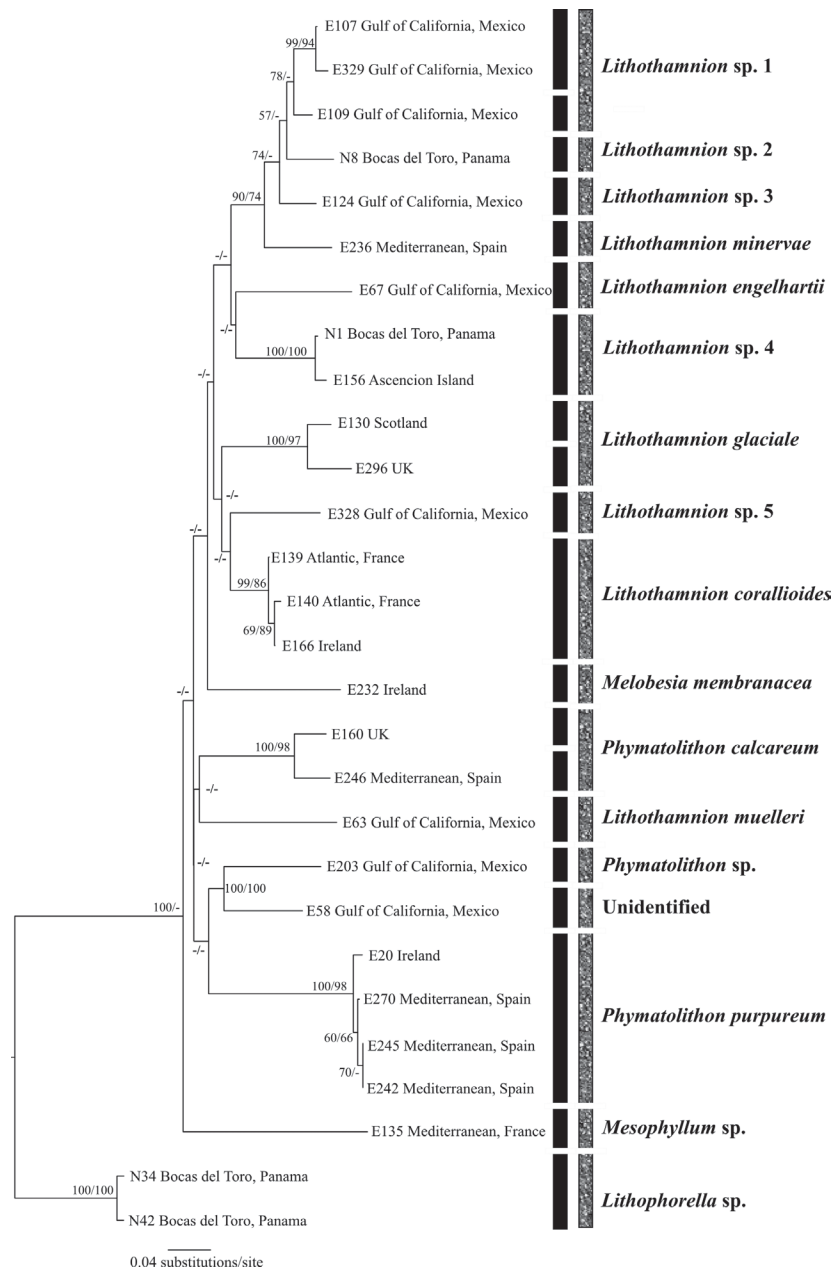
**TABLE 3.** Morphological differences among *Lithophyllum margaritae* A and B. *Lithophyllum margaritae* A is represented by specimen E53, which is identical to specimen E108 (haplotype 2). *Lithophyllum margaritae* B is represented by specimen E315 (haplotype 10) and E311 (haplotype 13).

	E53	E315	E311
Conceptacle surface diameter (µm)	220–250	375–500	440–460
Conceptacle transverse view, diameter (µm)	150–237	350–360	320
Conceptacle transverse view, height (µm)	100–137	175–180	170
Tetraspores pack diameter (µm)	27–37	20–24	-
Tetraspores pack height (µm)	75–80	50–60	-

### Melobesioideae

The NJ analysis for the subfamily Melobesioideae recovered similar topologies and bootstrap support as the ML, for which only the earliest is represented, including both bootstrap values. The taxa included algae morphologically referable to *Lithothamnion* Heydrich (1897: 412), *Phymatolithon* Foslie (1898: 4), *Melobesia* Lamouroux (1812: 186) and *Mesophyllum* Lemoine (1928: 251). Several clades corresponded well to certain morphological species, as in the case of *Lithothamnion glaciale* Kjellman (1883: 123), *L. corallioides*, *Phymatolithon calcareum*, and *P. purpureum* (Fig. 4). Only seven internal nodes out of 18 were moderately to highly supported, including values as 90/74 (samples E107, E329, E109, N8, E124, E236), 100/100 (samples N1, E156), 100/97 (samples E130, E296), 99/86 (samples E139, E140, E166), 100/98 (samples E160, E246), 100/100 (samples E203, E58) and 100/98 (samples E20, E270, E245, E242). Terminal nodes in the clades above mentioned presented moderate bootstrap values (e.g. *Phymatolithon purpureum* with 60/66,70/-). A clade recovered with moderate/high support (90/74) included specimens with a wide distribution range, including a specimen identified as *Lithothamnion minervae* Basso (1995: 354–358) from the Mediterranean (E236), an unidentified specimen from the Gulf of California (E124), an unidentified specimen from Panama (N8) and three unidentified specimens (E109, E329 and E107) from the Gulf of California. The maximum pairwise distance observed in this clade (between E236 and N8) was 13% (complete pairwise data in Supplementary Table 2). In contrast, the maximum pairwise distance among samples within the *Phymatolithon purpureum* clade was 1.62%, within the *Lithothamnion corallioides* clade was 0.6%, and within the *Phymatolithon calcareum* clade was 7.6%. The barcode gap and the two models used in the ABGD analysis (Jukes-Cantor [JC69] and Kimura [K80 TS/TV]) obtained identical results for the intra- and interspecific hypotheses (Fig. 4). Three incongruences were observed when comparing morpho-species and those results, subdividing *Lithothamnion* sp. 1 into two species, *Lithothamnion glaciale* into two species and *Phymatolithon calcareum* into two species. In the haplotype networks two haplotypes were present for *P. calcareum* (17 bp difference over an alignment of 335 bp, Fig. 5), one as the possible ancestor with three sequences all from the Mediterranean and the second haplotype represented by 27 sequences, all from Atlantic Europe including Ireland, UK, Spain and France. The species *Phymatolithon purpureum* was represented by four haplotypes, three from the Mediterranean (also the possible ancestor, sample E269 and E245) and a fourth from Ireland (samples E113, E20, E38 and E37); the divergence among the four haplotypes was up to 7 bp difference over an alignment of 301 bp (Fig. 5). Three haplotypes were found for *Lithothamnion corallioides* (up to two bp difference, Fig. 5); the possible ancestor was represented by six sequences from Ireland and France, while only France was represented in the remaining two

haplotypes (samples E140, E141 and E143; sample E139); the divergence among the three haplotypes was up to two basepairs over an alignment of 335 bp. *Phymatolithon purpureum* and *P. calcareum*, which both included samples from the Mediterranean, showed a similar haplotypic reconstruction with the Mediterranean haplotypes at the top and haplotypes from the North Atlantic (e.g. Ireland in the case of *Phymatolithon purpureum*) at the bottom.



**FIGURE 4.** Neighbour Joining analysis of the *cox2-3* spacer for members of the subfamily Melobesioideae. BP values are reported at nodes in the form Neighbor-Joining (NJ)/Maximum Likelihood (ML), respectively; nodes marked with '-' are not supported (>50%). Black bars demark the same hypotheses suggested by the p distance threshold and ABGD analysis using Jukes-Cantor (JC69) and Kimura (K80 TS/TV) models. Sand bar demarks the morphospecies based on anatomical examination and identification.

## Discussion

The *cox2-3* spacer was amplified successfully for taxa belonging to both subfamilies Lithophylloideae and Melobesioideae of the order Corallinales (Figs. 2 and 4). The lack of genus-level monophyly (Figs. 2 and 4) could be due to the high mutation rate in a marker generally used for population level diversity (Zuccarello *et al.* 1999, Couceiro *et al.* 2011). This pattern has also been observed in some markers used for DNA barcoding (e.g. ITS, *cox1*), which are generally saturated for reconstruction at higher taxonomic levels and require the support of a more conserved marker (Hajibabaei *et al.* 2007). Nevertheless, for both subfamilies (Fig. 2 and 4) the presence of some internally supported

branches could suggest the potential of the *cox2-3* spacer to group closely related species and/or populations instead of genera. It is also important to note that branches without support in our reconstructions could be due to incomplete taxon sampling, as it was suggested in previous studies (Hajibabaei *et al.* 2007).

The present study revealed some differences in pairwise distances for the *cox2-3* spacer due to the presence of gaps in the alignments. The barcode break found for each of the subfamilies varied considerably when including or excluding alignment gaps in the p distance analysis, but the total difference or the extent of the threshold was more stable (Table 2). Additionally, the barcode breaks obtained for the subfamilies Melobesioideae and Lithophylloideae were different (Table 2), this suggests that a universal threshold to be used as barcoding gap for the whole order Corallinales is unlikely to be detected. This is the first time values of intra- vs. interspecific variation have been given for this marker in coralline algae. This is valuable because it provides a reference to understand the divergence in the data set helping to discriminate the presence of multiple species.

The barcode break reported here for the Melobesioideae (1.9–4.7% and 1.9–3.8%) and Lithophylloideae (1.8–2.9% and 2.3–3.4%) provided a potential separation between species and population diversity and is moderate in comparison with high values for other red algal taxa. For example, *Ceramium tenuicorne* showed 0.4–4.0% of intra-specific variation, while among the species *C. tenuicorne*, *C. virgatum* Roth (1797: 148) and *Ceramium* sp. differences of 26.4–32.2% were recorded (Gabrielsen *et al.* 2002). However, lower values of interspecific variation were, for example, reported for *Peyssonnelia japonica* and *P. boudouresquei* (6.7%, Kato *et al.* 2009).

Specimens referred to *Lithophyllum margaritae* demonstrated high values of divergence for the A and B groups (Fig. 2, 17.3–23.2%). Besides, all the analyses used in this study (Fig. 2) suggested the presence of at least five species in group A and two species in group B. Only the separation of group A and B was corroborated by morphological means but no further morphological differences could be observed for the rest of the hypothetical species. Further collections are needed to reveal to what extent these haplotypes or hypothetical species are actually confirmed or rejected by synapomorphies, cross fertilization and geographical separation, among others.

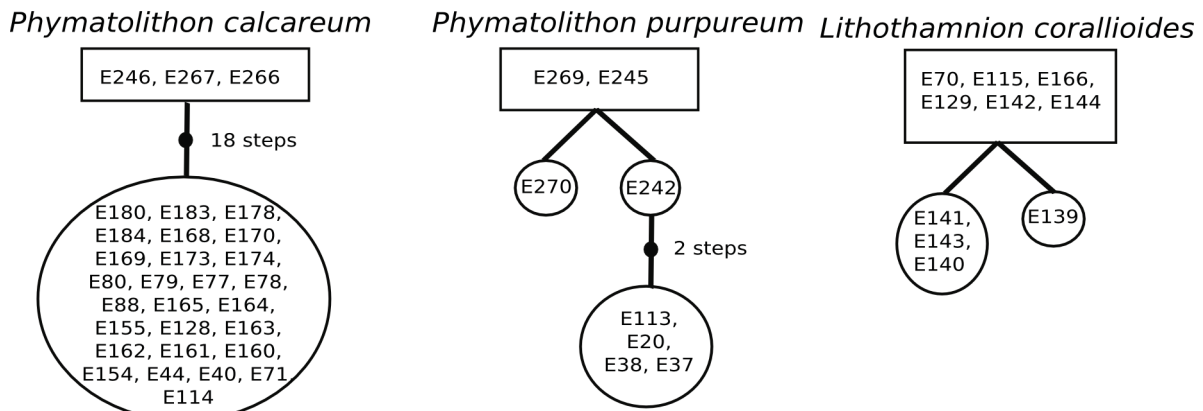
The results of the present study do not support the merge of the five entities in the Gulf of California into the single species *L. margaritae* proposed by Riosmena-Rodriguez *et al.* (1999). Furthermore, the molecular diversity of *L. margaritae* is considerably higher based on our results (Fig. 2) than previously suggested by only morphological means (Riosmena-Rodriguez *et al.* 1999). This is relevant in terms of conservation, because *Lithophyllum margaritae* has been reported as the most important rhodolith-forming species in the Gulf of California. Based on the present information a taxonomical revision is in progress, we are currently trying to acquire relevant type specimens and to sequence additional markers to resolve the number and identities of the species for each of the haplotypes found in the present study (Fig. 2).

Results in the present study suggested the presence of two possible species in the morphospecies *Phymatolithon calcareum*. Unfortunately, as for *L. margaritae*, the specimens did not present reproductive structures that could have helped to find synapomorphies. For that reason, in the present study both haplotypes (one from the Mediterranean and one from the North Atlantic) are treated as belonging to *P. calcareum* based on vegetative features traditionally reported for this species (Adey & McKibbin 1970) but with a high molecular divergence. Moreover, *P. purpureum* is reported here as a rhodolith in Europe and the Mediterranean, which has been reported traditionally as a thick crust and, in some rare conditions, as compact spheres (Irvine & Chamberlain 1994). Finally, the haplotype networks reconstructed for *P. calcareum* and *P. purpureum* (Fig. 5) showed similarities for the two geographical regions included, the Mediterranean Sea and Atlantic Europe, which could suggest that these two species had been shaped by the same evolutionary events in the area such glacial periods (Maggs *et al.* 2008).

Our results did not show any variation of the *cox2-3* spacer for *Phymatolithon calcareum* within a latitudinal range of 1300 km, from Northern Ireland to Galicia, Spain. The lack of variation of *Phymatolithon calcareum* in a wide geographical range using the *cox2-3* spacer may be the result of a high rate of clonal reproduction by vegetative fragmentation and a low rate of sexual reproduction in rhodolith-forming populations in NW Europe (see Cabioch 1970). Previous studies using the *cox1* and *psbA* genes and our results using the *cox2-3* spacer region suggest the possibility of long-range dispersal for coralline algae (Bittner *et al.* 2010). Additional molecular data and the inclusion of samples from other geographical regions (particularly around Gibraltar) should clarify the extent and borders of this population and the relationship with a hypothetical sibling species in the Mediterranean.

The presence of a well-supported clade including samples from the Mediterranean Sea (*Lithothamnion minervae*), the Gulf of California and Panama, suggests the presence of closely related taxa. The use of additional samples from the three regions and the use of other markers with different substitution rate should show a clear biogeographic pattern and relationships among these species in the genus *Lithothamnion*.

In conclusion, the *cox2-3* spacer appears to provide useful information in studies of phylogeography and barcoding analysis for related coralline taxa. The contrasting pattern observed for *Lithophyllum margaritae* in the Gulf of California and *Phymatolithon calcareum* in Europe suggests that any research or management approach for these two abundant and important rhodolith-forming species must be designed specifically for each one in their areas of distribution.



**FIGURE 5.** *Cox2-3* spacer haplotype networks for *Phymatolithon calcareum*, *P. purpureum* and *Lithothamnion corallioides*. Square box haplotype indicates the possible ancestral haplotype and a circle indicates a derived state. Two haplotypes were found for *Phymatolithon calcareum*; E246, E267 and E266 were specimens from the Mediterranean Sea, the rest were from northern Europe. Four haplotypes were found for *Phymatolithon purpureum*; E269, E245, E270 and E242 were specimens from the Mediterranean Sea, the rest were from northern Europe. Three haplotypes were found for *Lithothamnion corallioides*, all from northern Europe.

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**SUPPLEMENTARY MATERIAL TABLE 1.** Distance divergence per pair of sequences for the data set of *Lithophyllum margaritae*.

Haplotype 1	Haplotype 2	% Divergence
E57	E108	0.7
E57	E116	1.8
E108	E116	1.1
E57	E110	5.9
E108	E110	5.6
E116	E110	6.3
E57	E120	6.3
E108	E120	6.8
E116	E120	7.1
E110	E120	5.2
E57	E123	7.1
E108	E123	7.7
E116	E123	8.7
E110	E123	6.6
E120	E123	3.5
E57	E118	10.3
E108	E118	11.1
E116	E118	12.1
E110	E118	13.1
E120	E118	9.9
E123	E118	12.8
E57	E121	6.3
E108	E121	7.5
E116	E121	8.9
E110	E121	10.3
E120	E121	8.0
E123	E121	9.2
E118	E121	0.4
E57	E313	19.2
E108	E313	20
E116	E313	19.4
E110	E313	21.6
E120	E313	19.6
E123	E313	21.2
E118	E313	21.6
E121	E313	22.3
E57	E315	19.2
E108	E315	19.7
E116	E315	19.4
E110	E315	21.6
E120	E315	19.6
E123	E315	21.1
E118	E315	21.8
E121	E315	22.3
E313	E315	0.3
E57	E334	19.1
E108	E334	19.9
E116	E334	19.4
E110	E334	22.2
E120	E334	20.2
E123	E334	21.4
E118	E334	22.2
E121	E334	22.7
E313	E334	1.2
E315	E334	1.2
E57	E59	18.4

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**SUPPLEMENTARY MATERIAL TABLE 1. (Continued)**

Haplotype 1	Haplotype 2	% Divergence
E108	E59	18.2
E116	E59	17.8
E110	E59	23.1
E120	E59	20.3
E123	E59	21.5
E118	E59	22.1
E121	E59	22.5
E313	E59	6.8
E315	E59	7.0
E334	E59	7.5
E57	E311	17.7
E108	E311	18.2
E116	E311	17.6
E110	E311	21.9
E120	E311	19.9
E123	E311	20.2
E118	E311	20.5
E121	E311	20.3
E313	E311	7.2
E315	E311	7.4
E334	E311	7.8
E59	E311	2.9
E57	E52	18.7
E108	E52	19.3
E116	E52	18.7
E110	E52	23.2
E120	E52	20.2
E123	E52	21.0
E118	E52	20.1
E121	E52	19.8
E313	E52	9.0
E315	E52	9.2
E334	E52	9.7
E59	E52	4.0
E311	E52	1.0
E57	E64	18.0
E108	E64	18.4
E116	E64	17.9
E110	E64	22.3
E120	E64	19.6
E123	E64	20.7
E118	E64	17.3
E121	E64	19.3
E313	E64	8.4
E315	E64	8.4
E334	E64	9.1
E59	E64	3.2
E311	E64	0.3
E52	E64	0.3

**SUPPLEMENTARY MATERIAL TABLE 2.** Distance divergence per pair of sequences for the data set of Melobesioidea.

BT means Bocas del Toro, Panama.

Haplotype 1	Haplotype 2	% Divergence
1_BT	34_BT	34.8
1_BT	42_BT	38.8
34_BT	42_BT	1.2
1_BT	8_BT	18.1
34_BT	8_BT	35.0
42_BT	8_BT	38.6
1_BT	E107	17.0
34_BT	E107	34.2
42_BT	E107	38.2
8_BT	E107	7.3
1_BT	E109_CSL_GC	17.0
34_BT	E109_CSL_GC	33.9
42_BT	E109_CSL_GC	37.8
8_BT	E109_CSL_GC	8.3
E107_CSL_GC	E109_CSL_GC	4.7
1_BT	E124_REQUESON_GC	17.3
34_BT	E124_REQUESON_GC	33.4
42_BT	E124_REQUESON_GC	37.4
8_BT	E124_REQUESON_GC	9.6
E107_CSL_GC	E124_REQUESON_GC	7.1
E109_CSL_GC	E124_REQUESON_GC	5.1
1_BT	E130_LOCH_SWEEN_SCOTLAND	20.9
34_BT	E130_LOCH_SWEEN_SCOTLAND	36.6
42_BT	E130_LOCH_SWEEN_SCOTLAND	38.3
8_BT	E130_LOCH_SWEEN_SCOTLAND	22.4
E107_CSL_GC	E130_LOCH_SWEEN_SCOTLAND	21.4
E109_CSL_GC	E130_LOCH_SWEEN_SCOTLAND	21.4
E124_REQUESON_GC	E130_LOCH_SWEEN_SCOTLAND	22.3
1_BT	E135_MARSELLA_FRANCE	31.2
34_BT	E135_MARSELLA_FRANCE	36.4
42_BT	E135_MARSELLA_FRANCE	38.0
8_BT	E135_MARSELLA_FRANCE	31.7
E107_CSL_GC	E135_MARSELLA_FRANCE	30.3
E109_CSL_GC	E135_MARSELLA_FRANCE	31.7
E124_REQUESON_GC	E135_MARSELLA_FRANCE	30.1
E130_LOCH_SWEEN_SCOTLAND	E135_MARSELLA_FRANCE	31.8
1_BT	E139_BREST_FRANCE	17.3
34_BT	E139_BREST_FRANCE	32.7
42_BT	E139_BREST_FRANCE	35.9
8_BT	E139_BREST_FRANCE	16.9
E107_CSL_GC	E139_BREST_FRANCE	16.1
E109_CSL_GC	E139_BREST_FRANCE	14.6
E124_REQUESON_GC	E139_BREST_FRANCE	16.3
E130_LOCH_SWEEN_SCOTLAND	E139_BREST_FRANCE	14.1
E135_MARSELLA_FRANCE	E139_BREST_FRANCE	25.8
1_BT	E140_BREST_FRANCE	19.2
34_BT	E140_BREST_FRANCE	34.8
42_BT	E140_BREST_FRANCE	38.7
8_BT	E140_BREST_FRANCE	18.5
E107_CSL_GC	E140_BREST_FRANCE	17.0
E109_CSL_GC	E140_BREST_FRANCE	16.3
E124_REQUESON_GC	E140_BREST_FRANCE	17.9
E130_LOCH_SWEEN_SCOTLAND	E140_BREST_FRANCE	16.2
E135_MARSELLA_FRANCE	E140_BREST_FRANCE	25.8
E139_BREST_FRANCE	E140_BREST_FRANCE	0.7
1_BT	E156_ASCENSIONISLAND_ATLANTIC	1.2

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**SUPPLEMENTARY MATERIAL TABLE 2.** (Continued)

Haplotype 1	Haplotype 2	% Divergence
34_BT	E156_ASCENCIONISLAND_ATLANTIC	35.3
42_BT	E156_ASCENCIONISLAND_ATLANTIC	39.1
8_BT	E156_ASCENCIONISLAND_ATLANTIC	20.8
E107_CSL_GC	E156_ASCENCIONISLAND_ATLANTIC	18.8
E109_CSL_GC	E156_ASCENCIONISLAND_ATLANTIC	18.4
E124_REQUESON_GC	E156_ASCENCIONISLAND_ATLANTIC	18.3
E130_LOCH_SWEEN_SCOTLAND	E156_ASCENCIONISLAND_ATLANTIC	21.8
E135_MARSELLA_FRANCE	E156_ASCENCIONISLAND_ATLANTIC	31.2
E139_BREST_FRANCE	E156_ASCENCIONISLAND_ATLANTIC	17.9
E140_BREST_FRANCE	E156_ASCENCIONISLAND_ATLANTIC	18.3
1_BT	E160_NEOTYPE_CALCAREUM_UK	24.4
34_BT	E160_NEOTYPE_CALCAREUM_UK	32.9
42_BT	E160_NEOTYPE_CALCAREUM_UK	36.2
8_BT	E160_NEOTYPE_CALCAREUM_UK	25.4
E107_CSL_GC	E160_NEOTYPE_CALCAREUM_UK	22.8
E109_CSL_GC	E160_NEOTYPE_CALCAREUM_UK	22.8
E124_REQUESON_GC	E160_NEOTYPE_CALCAREUM_UK	24.2
E130_LOCH_SWEEN_SCOTLAND	E160_NEOTYPE_CALCAREUM_UK	25.4
E135_MARSELLA_FRANCE	E160_NEOTYPE_CALCAREUM_UK	32.1
E139_BREST_FRANCE	E160_NEOTYPE_CALCAREUM_UK	19.9
E140_BREST_FRANCE	E160_NEOTYPE_CALCAREUM_UK	20.8
E156_ASCENCIONISLAND_ATLANTIC	E160_NEOTYPE_CALCAREUM_UK	24.4
1_BT	E166_KILKIERAN_IRELAND	18.8
34_BT	E166_KILKIERAN_IRELAND	34.7
42_BT	E166_KILKIERAN_IRELAND	38.5
8_BT	E166_KILKIERAN_IRELAND	18.1
E107_CSL_GC	E166_KILKIERAN_IRELAND	17.3
E109_CSL_GC	E166_KILKIERAN_IRELAND	15.9
E124_REQUESON_GC	E166_KILKIERAN_IRELAND	17.5
E130_LOCH_SWEEN_SCOTLAND	E166_KILKIERAN_IRELAND	15.8
E135_MARSELLA_FRANCE	E166_KILKIERAN_IRELAND	26.3
E139_BREST_FRANCE	E166_KILKIERAN_IRELAND	0.4
E140_BREST_FRANCE	E166_KILKIERAN_IRELAND	0.3
E156_ASCENCIONISLAND_ATLANTIC	E166_KILKIERAN_IRELAND	17.9
E160_NEOTYPE_CALCAREUM_UK	E166_KILKIERAN_IRELAND	20.4
1_BT	E20_MUKINISH_IRELAND	28.9
34_BT	E20_MUKINISH_IRELAND	36.4
42_BT	E20_MUKINISH_IRELAND	39.3
8_BT	E20_MUKINISH_IRELAND	29.5
E107_CSL_GC	E20_MUKINISH_IRELAND	27.3
E109_CSL_GC	E20_MUKINISH_IRELAND	26.1
E124_REQUESON_GC	E20_MUKINISH_IRELAND	27.6
E130_LOCH_SWEEN_SCOTLAND	E20_MUKINISH_IRELAND	28.2
E135_MARSELLA_FRANCE	E20_MUKINISH_IRELAND	30.9
E139_BREST_FRANCE	E20_MUKINISH_IRELAND	22.1
E140_BREST_FRANCE	E20_MUKINISH_IRELAND	22.7
E156_ASCENCIONISLAND_ATLANTIC	E20_MUKINISH_IRELAND	27.2
E160_NEOTYPE_CALCAREUM_UK	E20_MUKINISH_IRELAND	26.1
E166_KILKIERAN_IRELAND	E20_MUKINISH_IRELAND	22.4
1_BT	E203_ISLAMARTIR_GC	22.8
34_BT	E203_ISLAMARTIR_GC	33.8
42_BT	E203_ISLAMARTIR_GC	37.6
8_BT	E203_ISLAMARTIR_GC	20.2
E107_CSL_GC	E203_ISLAMARTIR_GC	19.8
E109_CSL_GC	E203_ISLAMARTIR_GC	20.1
E124_REQUESON_GC	E203_ISLAMARTIR_GC	21.8
E130_LOCH_SWEEN_SCOTLAND	E203_ISLAMARTIR_GC	24.0

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**SUPPLEMENTARY MATERIAL TABLE 2. (Continued)**

Haplotype 1	Haplotype 2	% Divergence
E135_MARSELLA_FRANCE	E203_ISLAMARTIR_GC	30.1
E139_BREST_FRANCE	E203_ISLAMARTIR_GC	18.8
E140_BREST_FRANCE	E203_ISLAMARTIR_GC	21.0
E156_ASCENCIONISLAND_ATLANTIC	E203_ISLAMARTIR_GC	25.7
E160_NEOTYPE_CALCAREUM_UK	E203_ISLAMARTIR_GC	21.7
E166_KILKIERAN_IRELAND	E203_ISLAMARTIR_GC	20.6
E20_MUKINISH_IRELAND	E203_ISLAMARTIR_GC	23.2
1_BT	E232_FINAVARA_IRELAND	22.4
34_BT	E232_FINAVARA_IRELAND	36.2
42_BT	E232_FINAVARA_IRELAND	39.8
8_BT	E232_FINAVARA_IRELAND	21.6
E107_CSL_GC	E232_FINAVARA_IRELAND	19.9
E109_CSL_GC	E232_FINAVARA_IRELAND	19.9
E124_REQUESON_GC	E232_FINAVARA_IRELAND	20.8
E130_LOCH_SWEEN_SCOTLAND	E232_FINAVARA_IRELAND	23.9
E135_MARSELLA_FRANCE	E232_FINAVARA_IRELAND	30.5
E139_BREST_FRANCE	E232_FINAVARA_IRELAND	20.2
E140_BREST_FRANCE	E232_FINAVARA_IRELAND	21.6
E156_ASCENCIONISLAND_ATLANTIC	E232_FINAVARA_IRELAND	23.5
E160_NEOTYPE_CALCAREUM_UK	E232_FINAVARA_IRELAND	25.6
E166_KILKIERAN_IRELAND	E232_FINAVARA_IRELAND	21.2
E20_MUKINISH_IRELAND	E232_FINAVARA_IRELAND	25.9
E203_ISLAMARTIR_GC	E232_FINAVARA_IRELAND	22.5
1_BT	E236_BALEARICISLANDS_SPAIN	16.7
34_BT	E236_BALEARICISLANDS_SPAIN	35.9
42_BT	E236_BALEARICISLANDS_SPAIN	38.6
8_BT	E236_BALEARICISLANDS_SPAIN	11.7
E107_CSL_GC	E236_BALEARICISLANDS_SPAIN	9.0
E109_CSL_GC	E236_BALEARICISLANDS_SPAIN	10.0
E124_REQUESON_GC	E236_BALEARICISLANDS_SPAIN	11.0
E130_LOCH_SWEEN_SCOTLAND	E236_BALEARICISLANDS_SPAIN	23.7
E135_MARSELLA_FRANCE	E236_BALEARICISLANDS_SPAIN	32.6
E139_BREST_FRANCE	E236_BALEARICISLANDS_SPAIN	16.6
E140_BREST_FRANCE	E236_BALEARICISLANDS_SPAIN	18.7
E156_ASCENCIONISLAND_ATLANTIC	E236_BALEARICISLANDS_SPAIN	15.8
E160_NEOTYPE_CALCAREUM_UK	E236_BALEARICISLANDS_SPAIN	25.4
E166_KILKIERAN_IRELAND	E236_BALEARICISLANDS_SPAIN	18.2
E20_MUKINISH_IRELAND	E236_BALEARICISLANDS_SPAIN	29.3
E203_ISLAMARTIR_GC	E236_BALEARICISLANDS_SPAIN	21.7
E232_FINAVARA_IRELAND	E236_BALEARICISLANDS_SPAIN	23.7
1_BT	E245_BALEARICISLANDS_SPAIN	34.5
34_BT	E245_BALEARICISLANDS_SPAIN	42.6
42_BT	E245_BALEARICISLANDS_SPAIN	43.5
8_BT	E245_BALEARICISLANDS_SPAIN	34.9
E107_CSL_GC	E245_BALEARICISLANDS_SPAIN	33.5
E109_CSL_GC	E245_BALEARICISLANDS_SPAIN	32.0
E124_REQUESON_GC	E245_BALEARICISLANDS_SPAIN	33.0
E130_LOCH_SWEEN_SCOTLAND	E245_BALEARICISLANDS_SPAIN	33.6
E135_MARSELLA_FRANCE	E245_BALEARICISLANDS_SPAIN	31.8
E139_BREST_FRANCE	E245_BALEARICISLANDS_SPAIN	23.9
E140_BREST_FRANCE	E245_BALEARICISLANDS_SPAIN	25.9
E156_ASCENCIONISLAND_ATLANTIC	E245_BALEARICISLANDS_SPAIN	28.0
E160_NEOTYPE_CALCAREUM_UK	E245_BALEARICISLANDS_SPAIN	28.0
E166_KILKIERAN_IRELAND	E245_BALEARICISLANDS_SPAIN	25.8
E20_MUKINISH_IRELAND	E245_BALEARICISLANDS_SPAIN	1.6
E203_ISLAMARTIR_GC	E245_BALEARICISLANDS_SPAIN	30.0
E232_FINAVARA_IRELAND	E245_BALEARICISLANDS_SPAIN	32.9

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**SUPPLEMENTARY MATERIAL TABLE 2. (Continued)**

Haplotype 1	Haplotype 2	% Divergence
E236_BALEARICISLANDS_SPAIN	E245_BALEARICISLANDS_SPAIN	34.9
1_BT	E246_BALEARICISLANDS_SPAIN	27.2
34_BT	E246_BALEARICISLANDS_SPAIN	40.4
42_BT	E246_BALEARICISLANDS_SPAIN	41.1
8_BT	E246_BALEARICISLANDS_SPAIN	25.7
E107_CSL_GC	E246_BALEARICISLANDS_SPAIN	24.9
E109_CSL_GC	E246_BALEARICISLANDS_SPAIN	23.7
E124_REQUESON_GC	E246_BALEARICISLANDS_SPAIN	26.4
E130_LOCH_SWEEN_SCOTLAND	E246_BALEARICISLANDS_SPAIN	29.3
E135_MARSELLA_FRANCE	E246_BALEARICISLANDS_SPAIN	36.0
E139_BREST_FRANCE	E246_BALEARICISLANDS_SPAIN	24.1
E140_BREST_FRANCE	E246_BALEARICISLANDS_SPAIN	27.0
E156_ASCENCIONISLAND_ATLANTIC	E246_BALEARICISLANDS_SPAIN	29.8
E160_NEOTYPE_CALCAREUM_UK	E246_BALEARICISLANDS_SPAIN	7.6
E166_KILKIERAN_IRELAND	E246_BALEARICISLANDS_SPAIN	26.9
E20_MUKINISH_IRELAND	E246_BALEARICISLANDS_SPAIN	32.0
E203_ISLAMARTIR_GC	E246_BALEARICISLANDS_SPAIN	21.9
E232_FINAVARA_IRELAND	E246_BALEARICISLANDS_SPAIN	29.8
E236_BALEARICISLANDS_SPAIN	E246_BALEARICISLANDS_SPAIN	27.9
E245_BALEARICISLANDS_SPAIN	E246_BALEARICISLANDS_SPAIN	37.6
1_BT	E270_BALEARICISLANDS_SPAIN	28.9
34_BT	E270_BALEARICISLANDS_SPAIN	36.5
42_BT	E270_BALEARICISLANDS_SPAIN	39.0
8_BT	E270_BALEARICISLANDS_SPAIN	29.1
E107_CSL_GC	E270_BALEARICISLANDS_SPAIN	27.5
E109_CSL_GC	E270_BALEARICISLANDS_SPAIN	26.4
E124_REQUESON_GC	E270_BALEARICISLANDS_SPAIN	27.5
E130_LOCH_SWEEN_SCOTLAND	E270_BALEARICISLANDS_SPAIN	28.2
E135_MARSELLA_FRANCE	E270_BALEARICISLANDS_SPAIN	31.4
E139_BREST_FRANCE	E270_BALEARICISLANDS_SPAIN	23.6
E140_BREST_FRANCE	E270_BALEARICISLANDS_SPAIN	24.3
E156_ASCENCIONISLAND_ATLANTIC	E270_BALEARICISLANDS_SPAIN	27.4
E160_NEOTYPE_CALCAREUM_UK	E270_BALEARICISLANDS_SPAIN	25.7
E166_KILKIERAN_IRELAND	E270_BALEARICISLANDS_SPAIN	23.9
E20_MUKINISH_IRELAND	E270_BALEARICISLANDS_SPAIN	1.9
E203_ISLAMARTIR_GC	E270_BALEARICISLANDS_SPAIN	23.0
E232_FINAVARA_IRELAND	E270_BALEARICISLANDS_SPAIN	27.1
E236_BALEARICISLANDS_SPAIN	E270_BALEARICISLANDS_SPAIN	29.6
E245_BALEARICISLANDS_SPAIN	E270_BALEARICISLANDS_SPAIN	0.4
E246_BALEARICISLANDS_SPAIN	E270_BALEARICISLANDS_SPAIN	30.4
1_BT	E296_NEWFOUNDLAND	20.0
34_BT	E296_NEWFOUNDLAND	34.8
42_BT	E296_NEWFOUNDLAND	37.8
8_BT	E296_NEWFOUNDLAND	22.8
E107_CSL_GC	E296_NEWFOUNDLAND	22.1
E109_CSL_GC	E296_NEWFOUNDLAND	22.1
E124_REQUESON_GC	E296_NEWFOUNDLAND	22.1
E130_LOCH_SWEEN_SCOTLAND	E296_NEWFOUNDLAND	5.8
E135_MARSELLA_FRANCE	E296_NEWFOUNDLAND	31.8
E139_BREST_FRANCE	E296_NEWFOUNDLAND	15.0
E140_BREST_FRANCE	E296_NEWFOUNDLAND	17.1
E156_ASCENCIONISLAND_ATLANTIC	E296_NEWFOUNDLAND	21.2
E160_NEOTYPE_CALCAREUM_UK	E296_NEWFOUNDLAND	26.0
E166_KILKIERAN_IRELAND	E296_NEWFOUNDLAND	16.7
E20_MUKINISH_IRELAND	E296_NEWFOUNDLAND	28.1
E203_ISLAMARTIR_GC	E296_NEWFOUNDLAND	22.9
E232_FINAVARA_IRELAND	E296_NEWFOUNDLAND	23.6

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**SUPPLEMENTARY MATERIAL TABLE 2.** (Continued)

Haplotype 1	Haplotype 2	% Divergence
E236_BALEARICISLANDS_SPAIN	E296_NEWFOUNDLAND	24.2
E245_BALEARICISLANDS_SPAIN	E296_NEWFOUNDLAND	33.7
E246_BALEARICISLANDS_SPAIN	E296_NEWFOUNDLAND	29.3
E270_BALEARICISLANDS_SPAIN	E296_NEWFOUNDLAND	27.6
1_BT	E328_LITH_SANESTEBAN_GC	20.3
34_BT	E328_LITH_SANESTEBAN_GC	36.1
42_BT	E328_LITH_SANESTEBAN_GC	39.6
8_BT	E328_LITH_SANESTEBAN_GC	19.7
E107_CSL_GC	E328_LITH_SANESTEBAN_GC	17.7
E109_CSL_GC	E328_LITH_SANESTEBAN_GC	19.6
E124_REQUESON_GC	E328_LITH_SANESTEBAN_GC	19.5
E130_LOCH_SWEEN_SCOTLAND	E328_LITH_SANESTEBAN_GC	20.5
E135_MARSELLA_FRANCE	E328_LITH_SANESTEBAN_GC	30.5
E139_BREST_FRANCE	E328_LITH_SANESTEBAN_GC	13.0
E140_BREST_FRANCE	E328_LITH_SANESTEBAN_GC	14.9
E156_ASCENCIONISLAND_ATLANTIC	E328_LITH_SANESTEBAN_GC	21.5
E160_NEOTYPE_CALCAREUM_UK	E328_LITH_SANESTEBAN_GC	22.9
E166_KILKIERAN_IRELAND	E328_LITH_SANESTEBAN_GC	14.5
E20_MUKINISH_IRELAND	E328_LITH_SANESTEBAN_GC	25.4
E203_ISLAMARTIR_GC	E328_LITH_SANESTEBAN_GC	19.4
E232_FINAVARA_IRELAND	E328_LITH_SANESTEBAN_GC	18.6
E236_BALEARICISLANDS_SPAIN	E328_LITH_SANESTEBAN_GC	20.7
E245_BALEARICISLANDS_SPAIN	E328_LITH_SANESTEBAN_GC	32.6
E246_BALEARICISLANDS_SPAIN	E328_LITH_SANESTEBAN_GC	27.9
E270_BALEARICISLANDS_SPAIN	E328_LITH_SANESTEBAN_GC	25.6
E296_NEWFOUNDLAND	E328_LITH_SANESTEBAN_GC	20.3
1_BT	E329_SANESTEBAN_GC	18.0
34_BT	E329_SANESTEBAN_GC	34.2
42_BT	E329_SANESTEBAN_GC	39.0
8_BT	E329_SANESTEBAN_GC	8.3
E107_CSL_GC	E329_SANESTEBAN_GC	0.9
E109_CSL_GC	E329_SANESTEBAN_GC	5.7
E124_REQUESON_GC	E329_SANESTEBAN_GC	8.0
E130_LOCH_SWEEN_SCOTLAND	E329_SANESTEBAN_GC	22.0
E135_MARSELLA_FRANCE	E329_SANESTEBAN_GC	30.3
E139_BREST_FRANCE	E329_SANESTEBAN_GC	16.5
E140_BREST_FRANCE	E329_SANESTEBAN_GC	17.4
E156_ASCENCIONISLAND_ATLANTIC	E329_SANESTEBAN_GC	19.3
E160_NEOTYPE_CALCAREUM_UK	E329_SANESTEBAN_GC	23.0
E166_KILKIERAN_IRELAND	E329_SANESTEBAN_GC	17.7
E20_MUKINISH_IRELAND	E329_SANESTEBAN_GC	27.4
E203_ISLAMARTIR_GC	E329_SANESTEBAN_GC	20.1
E232_FINAVARA_IRELAND	E329_SANESTEBAN_GC	20.6
E236_BALEARICISLANDS_SPAIN	E329_SANESTEBAN_GC	10.0
E245_BALEARICISLANDS_SPAIN	E329_SANESTEBAN_GC	34.5
E246_BALEARICISLANDS_SPAIN	E329_SANESTEBAN_GC	26.1
E270_BALEARICISLANDS_SPAIN	E329_SANESTEBAN_GC	28.1
E296_NEWFOUNDLAND	E329_SANESTEBAN_GC	23.1
E328_LITH_SANESTEBAN_GC	E329_SANESTEBAN_GC	18.3
1_BT	E58_CSL_GC	23.5
34_BT	E58_CSL_GC	32.6
42_BT	E58_CSL_GC	35.0
8_BT	E58_CSL_GC	24.0
E107_CSL_GC	E58_CSL_GC	22.0
E109_CSL_GC	E58_CSL_GC	22.4
E124_REQUESON_GC	E58_CSL_GC	21.5
E130_LOCH_SWEEN_SCOTLAND	E58_CSL_GC	23.5

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**SUPPLEMENTARY MATERIAL TABLE 2. (Continued)**

Haplotype 1	Haplotype 2	% Divergence
E135_MARSELLA_FRANCE	E58_CSL_GC	29.4
E139_BREST_FRANCE	E58_CSL_GC	19.2
E140_BREST_FRANCE	E58_CSL_GC	21.8
E156_ASCENCIONISLAND_ATLANTIC	E58_CSL_GC	24.9
E160_NEOTYPE_CALCAREUM_UK	E58_CSL_GC	21.0
E166_KILKIERAN_IRELAND	E58_CSL_GC	21.4
E20_MUKINISH_IRELAND	E58_CSL_GC	22.1
E203_ISLAMARTIR_GC	E58_CSL_GC	14.6
E232_FINAVARA_IRELAND	E58_CSL_GC	23.9
E236_BALEARICISLANDS_SPAIN	E58_CSL_GC	23.2
E245_BALEARICISLANDS_SPAIN	E58_CSL_GC	28.5
E246_BALEARICISLANDS_SPAIN	E58_CSL_GC	24.0
E270_BALEARICISLANDS_SPAIN	E58_CSL_GC	23.0
E296_NEWFOUNDLAND	E58_CSL_GC	22.4
E328_LITH_SANESTEBAN_GC	E58_CSL_GC	19.4
E329_SANESTEBAN_GC	E58_CSL_GC	22.4
1_BT	E63_ELPARDITO_GC	27.2
34_BT	E63_ELPARDITO_GC	36.0
42_BT	E63_ELPARDITO_GC	39.4
8_BT	E63_ELPARDITO_GC	25.5
E107_CSL_GC	E63_ELPARDITO_GC	24.3
E109_CSL_GC	E63_ELPARDITO_GC	23.6
E124_REQUESON_GC	E63_ELPARDITO_GC	23.4
E130_LOCH_SWEEN_SCOTLAND	E63_ELPARDITO_GC	27.4
E135_MARSELLA_FRANCE	E63_ELPARDITO_GC	29.0
E139_BREST_FRANCE	E63_ELPARDITO_GC	18.8
E140_BREST_FRANCE	E63_ELPARDITO_GC	19.5
E156_ASCENCIONISLAND_ATLANTIC	E63_ELPARDITO_GC	27.1
E160_NEOTYPE_CALCAREUM_UK	E63_ELPARDITO_GC	25.4
E166_KILKIERAN_IRELAND	E63_ELPARDITO_GC	19.2
E20_MUKINISH_IRELAND	E63_ELPARDITO_GC	29.1
E203_ISLAMARTIR_GC	E63_ELPARDITO_GC	24.3
E232_FINAVARA_IRELAND	E63_ELPARDITO_GC	28.7
E236_BALEARICISLANDS_SPAIN	E63_ELPARDITO_GC	24.7
E245_BALEARICISLANDS_SPAIN	E63_ELPARDITO_GC	29.0
E246_BALEARICISLANDS_SPAIN	E63_ELPARDITO_GC	30.6
E270_BALEARICISLANDS_SPAIN	E63_ELPARDITO_GC	29.3
E296_NEWFOUNDLAND	E63_ELPARDITO_GC	26.2
E328_LITH_SANESTEBAN_GC	E63_ELPARDITO_GC	25.5
E329_SANESTEBAN_GC	E63_ELPARDITO_GC	25.3
E58_CSL_GC	E63_ELPARDITO_GC	23.6
1_BT	E67_CSL_GC	17.3
34_BT	E67_CSL_GC	35.0
42_BT	E67_CSL_GC	38.4
8_BT	E67_CSL_GC	20.6
E107_CSL_GC	E67_CSL_GC	17.1
E109_CSL_GC	E67_CSL_GC	16.7
E124_REQUESON_GC	E67_CSL_GC	18.5
E130_LOCH_SWEEN_SCOTLAND	E67_CSL_GC	23.0
E135_MARSELLA_FRANCE	E67_CSL_GC	28.0
E139_BREST_FRANCE	E67_CSL_GC	17.4
E140_BREST_FRANCE	E67_CSL_GC	18.0
E156_ASCENCIONISLAND_ATLANTIC	E67_CSL_GC	18.0
E160_NEOTYPE_CALCAREUM_UK	E67_CSL_GC	23.8
E166_KILKIERAN_IRELAND	E67_CSL_GC	17.6
E20_MUKINISH_IRELAND	E67_CSL_GC	27.7
E203_ISLAMARTIR_GC	E67_CSL_GC	26.3

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**SUPPLEMENTARY MATERIAL TABLE 2.** (Continued)

Haplotype 1	Haplotype 2	% Divergence
E232_FINAVARA_IRELAND	E67_CSL_GC	20.2
E236_BALEARICISLANDS_SPAIN	E67_CSL_GC	20.1
E245_BALEARICISLANDS_SPAIN	E67_CSL_GC	29.9
E246_BALEARICISLANDS_SPAIN	E67_CSL_GC	30.3
E270_BALEARICISLANDS_SPAIN	E67_CSL_GC	29.1
E296_NEWFOUNDLAND	E67_CSL_GC	25.8
E328_LITH_SANESTEBAN_GC	E67_CSL_GC	21.2
E329_SANESTEBAN_GC	E67_CSL_GC	17.4
E58_CSL_GC	E67_CSL_GC	26.7
E63_ELPARDITO_GC	E67_CSL_GC	25.3
1_BT	E242	35.0
34_BT	E242	42.2
42_BT	E242	43.5
8_BT	E242	35.4
E107_CSL_GC	E242	34.0
E109_CSL_GC	E242	32.7
E124_REQUESON_GC	E242	33.6
E130_LOCH_SWEEN_SCOTLAND	E242	33.4
E135_MARSELLA_FRANCE	E242	31.8
E139_BREST_FRANCE	E242	23.6
E140_BREST_FRANCE	E242	25.6
E156_ASCENCIONISLAND_ATLANTIC	E242	28.0
E160_NEOTYPE_CALCAREUM_UK	E242	27.6
E166_KILKIERAN_IRELAND	E242	25.5
E20_MUKINISH_IRELAND	E242	1.2
E203_ISLAMARTIR_GC	E242	30.3
E232_FINAVARA_IRELAND	E242	33.1
E236_BALEARICISLANDS_SPAIN	E242	35.7
E245_BALEARICISLANDS_SPAIN	E242	0.3
E246_BALEARICISLANDS_SPAIN	E242	38.4
E270_BALEARICISLANDS_SPAIN	E242	0.8
E296_NEWFOUNDLAND	E242	33.9
E328_LITH_SANESTEBAN_GC	E242	32.2
E329_SANESTEBAN_GC	E242	35.0
E58_CSL_GC	E242	29.2
E63	E242	29.4
E67	E242	29.5

**SUPPLEMENTARY MATERIAL FIGURE 1.** Pairwise distance for each pair analysed in Melobesioideae and Lithophylloideae with complete deletion of alignment gaps. Y-axis represents percentage of divergence while X-axis represents the rank detected in the data set. An arrow marks the threshold found and the divergence values.

