UNIVERSIDADE DA CORUÑA

Development and application of molecular markers to maerl-forming species in Europe: data for their conservation

PhD thesis

Cristina Pardo Carabias

2016

Departamento de Bioloxía Animal, Bioloxía Vexetal e Ecoloxía
Facultade de Ciencias

UNIVERSIDADE DA CORUÑA

Development and application of molecular markers to maerl-forming species in Europe: data for their conservation

Desarrollo y aplicación de marcadores moleculares a especies formadoras de maerl en Europa: datos para su conservación

Desenvolvemento e aplicación de marcadores moleculares a especies formadoras de maerl en Europa: datos para a súa conservación

PhD Thesis / Tesis de Doctorado / Tese de Doutoramento

Cristina Pardo Carabias

Supervisors / Directores / Directores:

Dr. Ignacio M. Bárbara Criado

Dr. Rodolfo Barreiro Lozano

Dr. Viviana Peña Freire

Tutor / Tutor / Titor:

Dr. Rodolfo Barreiro Lozano

Reviewed by / Revisado por / Revisado por:

Dr. Jacques Grall (Observatoire du Domaine Côtier de l'IUEM-OSU, France)

Dr. Rafael Riosmena Rodríguez (Universidad Autónoma de Baja California Sur, México)

Deposit / Depósito / Depósito: October / Octubre / Outubro 2015

Defense / Defensa / Defensa: January / Enero / Xaneiro 2016

Departamento de Bioloxía Animal, Bioloxía Vexetal e Ecoloxía. Facultade de Ciencias da UDC.

Programa Oficial de Doutoramento en Acuicultura (Interuniversitario). RD 1393/2007.

UNIVERSIDADE DA CORUÑA

IGNACIO M. BÁRBARA CRIADO, **RODOLFO BARREIRO LOZANO** AND **VIVIANA PEÑA FREIRE**, SENIOR LECTURER OF BOTANY, PROFESSOR OF ECOLOGY AND POSTDOCTORAL RESEARCHER, RESPECTIVELY, FROM DEPARTMENT OF ANIMAL BIOLOGY, PLANT BIOLOGY AND ECOLOGY OF THE UNIVERSITY OF A CORUÑA

CERTIFY:

That the following report entitled "**Development and application of molecular markers to maerl-forming species in Europe: data for their conservation**" written by Miss **CRISTINA PARDO CARABIAS** has been prepared under their supervision in the Department of Animal Biology, Plant Biology and Ecology at the Science Faculty of the University of A Coruña, within the framework of the Official PhD Program in Aquaculture (Inter) regulated by RD 1393/2007, and it meets the requirements to be defended and to aspire to the degree of "International PhD".

And for any legal statement, the present document is signed in A Coruña October 8, 2015.

Dr. Ignacio M. Bárbara Criado Dr. Rodolfo Barreiro Lozano Dr. Viviana Peña Freire

UNIVERSIDADE DA CORUÑA

IGNACIO M. BÁRBARA CRIADO, **RODOLFO BARREIRO LOZANO** Y **VIVIANA PEÑA FREIRE**, PROFESOR TITULAR DE BOTÁNICA, CATEDRÁTICO DE ECOLOGÍA Y CONTRATADA POSTDOCTORAL (XUNTA DE GALICIA MODALIDAD A), RESPECTIVAMENTE, DEL DEPARTAMENTO DE BIOLOGÍA ANIMAL, BIOLOGÍA VEGETAL Y ECOLOGÍA DE LA UNIVERSIDAD DE A CORUÑA

DECLARAN:

Que la siguiente memoria titulada "**Development and application of molecular markers to maerl-forming species in Europe: data for their conservation**" presentada por Doña **CRISTINA PARDO CARABIAS** ha sido realizada bajo su dirección en el Departamento de Biología Animal, Biología Vegetal y Ecología de la Facultad de Ciencias de la Universidad de A Coruña, dentro del Programa Oficial de Doctorado en Acuicultura (Interuniversitario) regulado por el RD 1393/2007, y cumple con las condiciones exigidas para ser defendido y optar al grado de "Doctora Internacional" ante el tribunal que lo deberá juzgar.

Y para que así conste a los efectos oportunos, firman la presente en A Coruña a 8 de Octubre de 2015.

Dr. Ignacio M. Bárbara Criado Dr. Rodolfo Barreiro Lozano Dra. Viviana Peña Freire

UNIVERSIDADE DA CORUÑA

IGNACIO M. BÁRBARA CRIADO, **RODOLFO BARREIRO LOZANO** E **VIVIANA PEÑA FREIRE**, PROFESOR TITULAR DE BOTÁNICA, CATEDRÁTICO DE ECOLOXÍA E CONTRATADA POSDOUTORAL (XUNTA DE GALICIA MODALIDADE A), RESPECTIVAMENTE, DO DEPARTAMENTO DE BIOLOXÍA ANIMAL, BIOLOXÍA VEXETAL E ECOLOXÍA DA UNIVERSIDADE DA CORUÑA

DECLARAN:

Que a seguinte memoria titulada "**Development and application of molecular markers to maerl-forming species in Europe: data for their conservation**" presentada por Dona **CRISTINA PARDO CARABIAS** foi realizada baixo a súa dirección no Departamento de Bioloxía Animal, Bioloxía Vexetal e Ecoloxía da Facultade de Ciencias da Universidade da Coruña, dentro do Programa Oficial de Doutoramento en Acuicultura (Interuniversitario) regulado polo RD 1393/2007, e cumpre coas condicións esixidas para ser defendido e optar ao grao de "Doutora Internacional" ante o tribunal que o deberá xulgar.

E para que así conste aos efectos oportunos, asinan a presente na Coruña a 8 de Outubro de 2015.

Dr. Ignacio M. Bárbara Criado Dr. Rodolfo Barreiro Lozano Dra. Viviana Peña Freire

This PhD thesis was funded by the projects CTM2010-18787 (Ministerio de Ciencia e Innovación del Gobierno de España, partially funded by ERDF-the European Regional Development Fund-) and 10MMA103003PR (Xunta de Galicia). Sampling in Brittany (France) was supported by a European Community-Research Infrastructure Action under the FP7 ''Capacities'' Specific Programme (ASSEMBLE grant agreement no. 227799).

Cristina Pardo Carabias appreciates the fellowships received to carry out the present PhD thesis in "Universidade da Coruña":

- "*Axuda de apoio á etapa predoutoral do Plan Galego de Investigación, Innovación e Crecemento 2011-2015 (Plan I2C) para o ano 2011 da Consellería de Cultura, Educación e Ordenación Universitaria da Xunta de Galicia. Expediente PRE/2011/058*" from November 1st, 2011 to December 20th, 2012.

- "*Beca del Programa de Formación del Profesorado Universitario (FPU). Convocatoria 2010. Nuevas becas del Ministerio de Educación del Gobierno de España. Referencia AP2010-1682*" from December 21st, 2012 to October 31st, 2015.

Cristina Pardo Carabias also appreciates a scholarship to carry out a research stay in the "Station Biologique de Roscoff" (France) from September 15th 2014 to 15th December 2014 under the supervision of Dr. Myriam Valero:

- "*Ayuda del Programa de Formación del Profesorado Universitario (FPU). Convocatoria 2013. Estancias breves del Ministerio de Educación, Cultura y Deporte del Gobierno de España. Referencia EST13/00886*".

To my parents, my sisters and my nephew, who have always been there.

A mis padres, mis hermanas y mi sobrino, que siempre han estado ahí.

Aos meus pais, as miñas irmás e o meu sobriño, que sempre estiveron aí.

ACKNOWLEDGMENTS

I would like to thank my PhD supervisors Dr. Ignacio M. Bárbara, Dr. Rodolfo Barreiro and Dr. Viviana Peña for their inestimable support and help during this thesis.

I want to thank all my partners of the BIOCOST Research Group for their help, support and shared moments during this thesis: Javier Cremades, Pilar Díaz, Juan Lugilde, Alicia García, Verónica García, J. Miguel Ruíz, Sergio R. Roiloa, Cristina Piñeiro, Pablo Touriñán, José Ribamar, María Quintela, Lucía Couceiro, Naiara Albaina and Isabel Maneiro. In addition, I want to thank Belén Carro and Lúa López that without their inestimable help and support, this thesis would not have been possible.

I am grateful to Dr. Myriam Valero for welcoming me in her Research Group during my stay in Roscoff (Brittany) and all people of her Research Group. Also thanks to all people that during my stay encouraged and help me: Alejandro Montecinos, Katherine Valenzuela, Zujaila Qui Minet, Marie Rescan, Jérome Coudret, Komlan Avia and Marie-Laure Guillemin. I am also grateful to Dr. Christophe Destombe for his invaluable advice and for giving me the chance to meet Dr. Jacqueline Cabioch. Madame Cabioch, thanks for sharing with me your knowledge and by your great advices in maerl beds.

My friends and University partners have always supported and inspired me: Laura Sanjurjo, Rafael Carballeira, Teresa Martínez, Esther Novo, Tamara Rábade, Tania García, Marta Lois, Ana B. Monteagudo, Alba García and Irene Lema. In addition, I would like to thank my old friends Raquel Abal, Iria Rodríguez, Miguel Delgado and Fátima Prol for all the moments that they shared with me, thanks guys. Besides, I would like to highlight the company, support and help from my comrade Javier Veloso during my thesis journey and life.

Finally, I thank my family for their company and support: my parents Mercedes and Emilio J., my sisters Mónica and Rosa, my nephew Hugo, my uncles Teresa and Luís, my cousins Silvia and Fernando, my godparents Suso and Ana, and my grandparents Emilia and Delfín that although they are no longer here they taught me the best of life.

ABSTRACT

Appropriate management of habitats of conservation concern, such as the maerl beds, requires reliable information on basic issues like species diversity, life cycle, and the population genetics of maerl-forming species. Conventionally, these topics had been addressed with morphological information. In comparison, the present thesis has tried to step forward by using molecular markers to solve these gaps in our knowledge. In chapter 1, maerl-forming species were delimited in Atlantic Europe using an integrative taxonomic approach that included COI-5P and *psb*A sequences as molecular data. A new major maerl-forming species was discovered by DNA barcodes, and its description is provided in chapter 2. A contribution to disentangle the life cycle of a major maerl-forming species (*Phymatolithon calcareum*) was obtained with a combination of DNA barcodes and morphology in chapter 3. Chapter 4 describes the development of a set of microsatellite markers for this species using NGS technology. Finally, preliminary results obtained with these microsatellites to several populations of *P. calcareum* across European Atlantic maerl beds are shown in a final Annex to this thesis.

RESUMEN

La gestión adecuada de los hábitats de interés para la conservación, como los bancos de maerl, requiere información previa sobre sus aspectos básicos, como la diversidad específica, los ciclos de vida y la genética de poblaciones. Convencionalmente, estos aspectos habían sido abordados con información morfológica en los bancos de maerl. Esta tesis ha tratado de dar un paso adelante usando marcadores moleculares para resolver estas cuestiones. En el capítulo 1, las especies formadoras de maerl del Atlántico Europeo fueron delimitadas utilizando un enfoque taxonómico integrador, incluyendo secuencias de COI-5P y *psb*A. También se ha descrito una nueva especie formadora de maerl mayoritaria a través del uso de códigos de barras de ADN (capítulo 2). Además, en el capítulo 3, se ha realizado una contribución sobre el ciclo de vida de una de las principales especies formadoras de maerl (*Phymatolithon calcareum*) mediante la combinación de información molecular y morfológica. En el capítulo 4 se ha descrito el desarrollo de marcadores microsatélite para esta especie utilizando la tecnología NGS. Finalmente, en un anexo final de esta tesis, se muestran los resultados preliminares obtenidos tras la aplicación de estos microsatélites a varias poblaciones de *P. calcareum* en fondos de maerl del Atlántico Europeo.

RESUMO

A xestión adecuada dos hábitats de interese para a conservación, como os bancos de maerl, require información previa sobre os seus aspectos básicos, como a diversidade específica, os ciclos de vida e a xenética de poboacións. Convencionalmente, estes aspectos foran abordados con información morfolóxica nos bancos de maerl. Esta tese tratou de dar un paso adiante usando marcadores moleculares para resolver estas cuestións. No capítulo 1, as especies formadoras de maerl do Atlántico Europeo foron delimitadas utilizando un enfoque taxonómico integrador, incluíndo secuencias de COI-5P e *psb*A. Tamén se describiu unha nova especie formadora de maerl maioritaria a través do uso de códigos de barras de ADN (capítulo 2). Ademáis, no capítulo 3, fíxose unha contribución sobre o ciclo de vida dunha das principais especies formadoras de maerl (*Phymatolithon calcareum*), mediante a combinación de información molecular e morfolóxica. No capítulo 4 describiuse o desenvolvemento de marcadores microsatélite para *P. calcareum* utilizando a tecnoloxía NGS. Finalmente, nun anexo final desta tese, móstranse os resultados preliminares obtidos tras a aplicación destes microsatélites a varias poboacións de *P. calcareum* en fondos de maerl do Atlántico Europeo.

INDEX

ABBREVIATIONS

ABC= Approximate Bayesian Computation

ABGD= Automatic Barcode Gap Discovery

A_c= spatial aggregation index for clonal aggregation

AFLPs= Amplified Fragment Length Polymorphisms

AIC= Akaike Information Criterion

Allozymes= Allelic variants of enzymes

BEAST= Bayesian Evolutionary Analysis by Sampling Trees

BEST= Bayesian Estimation of Species Trees

BF= Bayes Factor

BFD= Bayes Factor Delimitation

bGMYC= Bayesian General Mixed Yule Coalescent model

BI= Bayesian Inference

BIC= Bayesian Information Criterion

BINs= Barcode Index Numbers

bp= base pairs

BP&P = Bayesian Phylogenetics & Phylogeography

BSC= Biological Species Concept

CCD= Charge-Coupled Device

COI-5P= 5' of the mitochondrial gene Cytochrome c Oxidase subunit I

cox2: gene Cytochrome c Oxidase subunit 2

cox2-3: partial genes *cox*2 (Cytochrome c Oxidase subunit 2) and *cox*3 (Cytochrome c Oxidase subunit 3) and intergenic spacer

CS= Clonal Subrange

*D**= adapted Simpson's index for clonal geotypic diversity to estimate the clonal heterogeneity

D_{max}= minimum value of adapted Simpson's index for genotypic diversity

D_{min}= maximum value of adapted Simpson's index for genotypic diversity

dNTP= deoxyriboNucleoside TriPhosphate

EcSC= Ecological Species Concept

*ED**= clonal evenness index of Simpson

EF2= Elongation Factor 2 gene

emPCR= emulsion PCR

EUNIS = European Nature Information System

F_{IS}= inbreeding coefficient

F-statistics= Fixation-statistics

G= genotypic richness

GCSC= Genotypic Cluster Species Concept

GMYC= General Mixed Yule Coalescent model

GTR+G=Generalized Time-Reversible model with Gamma distribution

GTR+G+I=Generalized Time-Reversible model with Gamma distribution plus Invariant sites

H_E= Expected Heterozygosity

HKY+G= Hasegawa-Kishino-Yano model with Gamma distribution

HME= Harmonic Mean Estimation

H_O= Observed Heterozygosity

H-W= Hardy-Weinberg

ICN= International Code of Nomenclature

ITS= Internal Transcribed Spacer of the ribosomal cistron

IUCN = International Union for Conservation of Nature

K2P= Kimura two Parameters

L_0= Likelihood of the null model

LD= Linkage Disequilibrium

$L_{multiple}$= Likelihood of the multiple-threshold of GMYC model

L_{single}= Likelihood of the single-threshold of GMYC model

LSU= Large SubUnit of 28S rRNA gene

MCMC= Markov Chain Monte Carlo

ML= Maximum Likelihood

MLGs= MultiLocus Genotypes

MLL= MultiLocus Lineage

MSC= Morphological Species Concept

n= sample size as number of successful DNA amplifications

N= Number of individuals

N_A= Number of observed Alleles

N-J= Neighbor-Joining

NGS= Next Generation Sequencing

N_I= number of DNA samples (individuals) assayed

OSPAR= Oslo and Paris Convention, currently named as "The Convention for the Protection of the Marine Environment of the North-East Atlantic"

PCR= Polymerase Chain Reaction

Phycoerythrin gene: alpha (*cpe*A) and beta (*cpe*B) subunits and an intervening spacer

p_i^2= frequency of the *i*th MLL

PIC= Polymorphism Information Content

PS= Path Sampling

*psb*A= plastidial gene photosystem II reaction center protein D1

PSC= Phylogenetic Species Concept

PSHs= Primary Species Hypotheses

PTP= Poisson Tree Processes

PTPWs= PicoTiterPlate Wells

QTLs= Quantitative Trait Loci

R= index of clonal diversity

RAD-seq= Restriction-site-Associated DNA sequencing

RAPDs= Random Amplified Polymorphic DNAs

*rbc*L= Large subunit of Ribulose-1,5-Biphosphate Carboxylase oxygenase

RFLPs= Restriction Fragment Length Polymorphisms

RuBisCo spacer: partial *rbc*L (Large subunit of Ribulose-1,5-Biphosphate Carboxylase oxygenase) and *rbc*S (Small subunit of Ribulose-1,5-Biphosphate Carboxylase oxygenase) genes with intergenic spacer

SEM= Scanning Electron Microscope

SNPs= Single Nucleotide Polymorphisms

SpedeSTEM = SPEecies DElimitation with Species Tree Estimation using Maximum likelihood

SPIDER: SPecies IDentity and Evolution in R

SS= Steppin-stone Sampling

SSHs= Secondary Species Hypotheses

SSRs= Simple Sequence Repeats

SSU= Small SubUnit of 18S rRNA gene

STEM= Species Tree Estimation using Maximum likelihood

STRs = Short Tandem Repeats

STSs= Seguence-Tagged Sites

UCLN= UnCorrelated Log Normal

UPA= Universal Plastid Amplicon of 23S rRNA gene

UPC= Universal Product Code

454 NGS= 454 GS-FLX Next Generation Sequencing

INTRODUCTION

1. Conservation Biology: protecting species

The natural world is a far different place now than it was 10,000 years ago, or even 100 years ago. Humans have altered every natural ecosystem on the planet, some to the point of collapse. Huge numbers of species have gone prematurely extinct, natural hydrologic and chemical cycles have been disrupted, billions of tons of topsoil have been lost, genetic diversity has eroded, and the very climate of the planet may have been disrupted. The cause of such vast environmental change is the cumulative impacts of people that have stressed the ecological support systems of the planet past their powers of resilience. As a consequence, biological diversity (i.e. biodiversity) the grand result of evolutionary processes and events tracing back several billion years is itself at stake and rapidly declining. In this context, the discipline of Conservation Biology (**Fig. 1**) has been born as a response of the scientific community to the biodiversity crisis (Meffe & Carroll 1994).

Conservation Biology addresses the biology of species, communities, and ecosystems that are perturbed, either directly or indirectly, by human activities or other agents, and its goal is to provide principles and tools for preserving biological diversity (Soulé 1985). Biodiversity refers to the variability among living organisms from all sources including terrestrial, marine and other aquatic ecosystems, and the ecological complexes of which they are part; this comprises ecosystem diversity, species diversity and genetic diversity (International Union for Conservation of Nature, IUCN, see http://iucn.org/iyb/about/, McNeely *et al.* 1990).

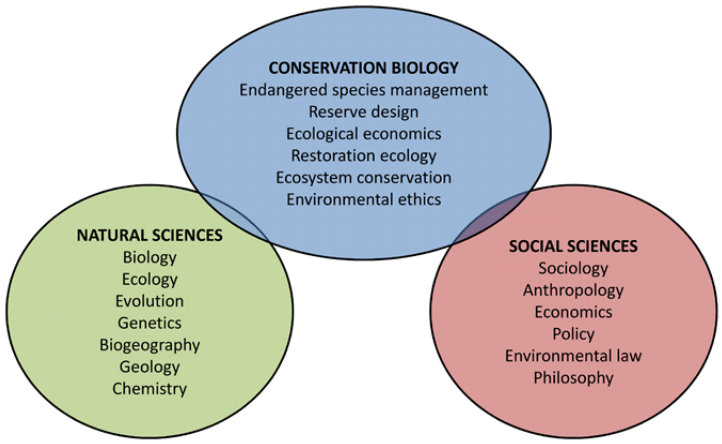


Fig. 1. The interdisciplinary nature of Conservation Biology merges many traditional fields of natural and social sciences. The list of relevant sub-disciplines and interactions shown is not meant to be exhaustive. From Meffe & Carroll (1994).

Preserving biological diversity requires an appropriate knowledge of the populations of the various species that live together in time and space. Therefore, to conserve biodiversity, it is first necessary to define the term "species". There is a lack of consensus on what is a "species" as illustrated by the range of definitions that have been proposed (**Table 1**; de Queiroz 2007).

From a biological point of view (Biological Species Concept, BSC), species are groups of actually or potentially interbreeding natural populations, which are reproductively isolated from similar groups thus protecting the integrity of their genotypes (Mayr 1942, 1996, de Queiroz 2005). The ecological approach (Ecological Species Concept, EcSC) sustains that each species is defined by the adaptative zone or ecological niche that occupies (Van Valen 1976, Andersson 1990). On the other hand, species are monophyletic groups of organisms (i.e. consisting of an antecesor and all of its descendants, commonly inferred from possession of shared derived character states, Donoghue 1985, Mishler 1985) from a phylogenetic perspective (Phylogenetic Species Concept, PSC), or the smallest groups that are consistently and persistently distinguishable by ordinary means when appling a morphological (phenetic) approach (Morphological Species Concept, MSC) (Cronquist 1978). In comparison, the Genotypic Cluster Species Concept (GCSC) states that species can be defined by genotypic clusters which are recognized by a deficit of genetic intermediates, both at single (heterozygote deficits) and multiple loci (strong correlations or disequilibrium between loci that are divergent between clusters). Mendelian variation within these clusters is discrete; therefore, quantized differences between individuals are expected. Patterns of the discrete genetic differences are used, rather than the discreteness itself, to reveal genotypic clusters

Table 1. Alternative species concepts and the properties on which they are based. Modified from de Queiroz (2007).

Species concept	Property	Reference
Biological (BSC)	Interbreeding (natural reproduction resulting in viable and fertile offspring). Intrinsic reproductive isolation (absence of interbreeding between heterospecific organisms based on intrinsic properties, as opposed to extrinsic —geographic—barriers)	Mayr (1942,1996)
Ecological (EcSC)	Same niche or adaptive zone (all components of the environment with which conspecific organisms interact)	Van Valen (1976) Andersson (1990)
Phylogenetic (PSC)	Monophyly	Donoghue (1985) Mishler (1985)
Morphological (MSC)	Recognition of groups of morphologically similar individuals	Cronquist (1978)
Genotypic Cluster (GCSC)	Form a genotypic cluster	Mallet (1995)

(Mallet 1995). Despite the lack of consensus, it is widely acknowledged that species arise by a speciation process when isolation of populations by interrupted gene flow leads to divergence due to selection and drift, and ultimately to separately evolving lineages that form new species (**Fig. 2**; Leliaert *et al.* 2014).

In the particular case of algae, delimiting species using morphology and/or sexual compatibility is challenging at best (Leliaert *et al.* 2014). Particularly in red algae (Rhodophyta), where phenotypic plasticity is widespread, life cycle is only partially known for many species, and a shared ecological distribution is frecuent (Irvine & Chamberlain 1994), PSC and GCSC are seem the definitions of "species" that fit best this type of organism. Interestingly, both PSC and GCSC depend on molecular information. In this context, several molecular methods have been developed to test hypotheses of lineage separation (i.e. delimiting species and testing traditional species boundaries) and to define species, changing our understanding of species boundaries (Sites & Marshall 2003, Carstens *et al.* 2013, Leliaert *et al.* 2014).

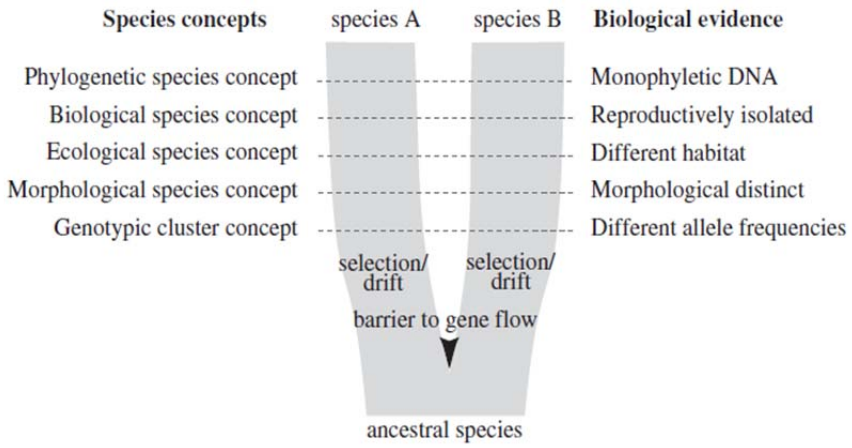


Fig 2. Simplified diagram of speciation, species concepts and corresponding biological properties of species. As populations separate by a barrier to gene flow, independently acting selection and drift will result in two daughter linages with separate evolutionary trajectories. Through time, daughter lineages will acquire different properties, which have traditionally served as biological evidence for species delimitation, corresponding to different species concepts. During the process of speciation, these secondary properties do not necessarily arise at the same time or in a regular order, and therefore different species concepts may come into conflict, especially during early stages of speciation. Modified from Leliaert *et al.* (2014).

1.1. Measuring species diversity with a molecular approach

DNA-based methods for the assessment of species diversity can be divided in single- and multi-locus approaches. However, it should be noted that this subdivision is somewhat artificial because several techniques fall into both categories (Leliaert *et al.* 2014).

DNA barcoding is the most popular approach in the single-locus category (http://www.ibol.org; Ratnasingham & Hebert 2007). This technique employs short fragments of DNA from a standardized region of the genome to identify species, in the same way a supermarket scanner uses the familiar black stripes of the UPC (Universal Product Code) barcode to identify your shopping articles (Hebert *et al.* 2003,2004, Saunders 2005, Robba *et al.* 2006). DNA barcodes are also used to detect cryptic species (e.g. Kucera & Saunders 2012, Milstein & Saunders 2012) and to help in the discovery of new species (e.g. Hind & Saunders 2013a,b). They have been widely used in Rhodophyta, where they are usually consist on sequences of the 5' end of the mitochondrial gene cytochrome c oxidase subunit I (COI-5P, also called *cox*1; Saunders 2005, Saunders & McDevit 2012b), although other DNA regions had been also used as DNA barcodes in red algae (**Table 2**).

A major difficulty with DNA barcodes is the need to discriminate interspecific divergence (the target for species delimitation) from intraspecific variation. An approach is the analysis of the frequency distribution of pairwise genetic distances when the data set includes conspecifics as well as representatives of several species. In an ideal situation, the transition between intra- and inter-specific variation should be denoted by a distinguishable "gap" in the distribution (i.e. "barcode gap" or "barcoding gap", **Fig. 3**), and a corresponding distance threshold could then be applied to delimit species (Hebert *et al.* 2004). However, intra- and inter-specific variations often overlap when the data set includes many closely related taxa, rendering the threshold approach problematic (**Fig. 3**; Moritz & Cicero 2004, Meyer & Paulay 2005).

Table 2. Markers used for DNA-based species delimitation and/or barcoding in red algae. COI-5P (i.e. *cox*1): 5' end of gene Cytochrome c Oxidase subunit I. *cox*2: gene Cytochrome c Oxidase subunit 2. *cox*2-3: partial genes *cox*2 and *cox*3 (Cytochrome c Oxidase subunit 3) and intergenic spacer. Phycoerythrin gene: alpha (*cpe*A) and beta (*cpe*B) subunits and an intervening spacer. *pbs*A: plastidial gene encoding protein D1 of the photosystem II reaction center. *rbc*L: Large subunit of Ribulose-1,5-Biphosphate CarboxyLase oxygenase. RuBisCo spacer: partial *rbc*L (Large subunit of Ribulose-1,5-Biphosphate Carboxylase oxygenase) and *rbc*S (Small subunit of Ribulose-1,5-biphosphate Carboxylase oxygenase) genes with intergenic spacer. UPA*:* Universal Plastid Amplicon of 23S rRNA gene. EF2: Elongation Factor 2 gene. ITS: Internal Transcribed Spacer of the ribosomal cistron. LSU: Large SubUnit of 28S rRNA gene. SSU: Small SubUnit of 18S rRNA gene. Underlined references are studies that include non-geniculate coralline red algae.

Type of marker	Marker	References
Mitochondrial	COI-5P (i.e. *cox*1)	Saunders (2005, 2009); Robba *et al.* (2006); Sherwood *et al.* (2008); Yang *et al.* (2008); Conklin *et al.* (2009); Clayden & Saunders (2010, 2014); Clarkston & Saunders (2010, 2012); Le Gall & Saunders (2010a); Manghisi *et al.* (2010); Rueness (2010); Sherwood *et al.* (2010a,b); Wolf *et al.* (2011); Costa *et al.* (2012); Genovese *et al.* (2012); Kucera & Saunders (2012); Milstein & Saunders (2012); Saunders & McDevit (2012a, 2013); Tan *et al.* (2012); Vis *et al.* (2012); Dixon & Saunders (2013); Hind & Saunders (2013a); Janouškovec *et al.* (2013); Kim *et al.* (2013); Necchi *et al.* (2013); Yang *et al.* (2013a,b); Agostinho & Necchi (2014); Carro *et al.* (2014); Conklin *et al.* (2014); Hind *et al.* (2014b); Nauer *et al.* (2014); Preuss & Zuccarello (2014); Salomaki *et al.* (2014); Vergés *et al.* (2014); de Jesus *et al.* (2015); Guoying *et al.* (2015); Lyra *et al.* (2015); Pardo *et al.* (2015a); Yang & Kim (2015)
	*cox*2	Tan *et al.* (2012)
	*cox*2-3	Conklin *et al.* (2009); Tan *et al.* (2012); Payo *et al.* (2013); Hernández-Kantún *et al.* (2014); Preuss & Zuccarello (2014)
Plastidial	Phycoerythrin gene	Yang & Boo (2006)
	*psb*A	Hind & Saunders (2013a); Carro *et al.* (2014); Hind *et al.* (2014b); Sissini *et al.* (2014)
	*rbc*L	Yang *et al.* (2008); Wolf *et al.* (2011); Costa *et al.* (2012); Geoffroy *et al.* (2012); Kucera & Saunders (2012); Saunders & McDevit (2012a); Tan *et al.* (2012); Vis *et al.* (2012); Dixon & Saunders (2013); Janouškovec *et al.* (2013); Kim *et al.* (2013); Necchi *et al.* (2013); Payo *et al.* (2013); Yang *et al.* (2013b); Agostinho & Necchi (2014); Conklin *et al.* (2014); Manghisi *et al.* (2014); Nauer *et al.* (2014); Salomaki *et al.* (2014); Sissini *et al.* (2014); Lyra *et al.* (2015); de Jesus *et al.* (2015); Yang & Kim (2015)
	RuBisCo spacer	Brodie *et al.* (1996, 1998); Robba *et al.* (2006); Hind *et al.* (2014b); Preuss & Zuccarello (2014)
	UPA	Presting (2006); Sherwood *et al.* (2008); Conklin *et al.* (2009); Clarkston & Saunders (2010, 2012); Sherwood *et al.* (2010a,b); Costa *et al.* (2012); Guoying *et al.* (2015); Kucera & Saunders (2012); Milstein & Saunders (2012); Saunders & McDevit (2012a); Vis *et al.* (2012); Nauer *et al.* (2014); Sissini *et al.* (2014); Lyra *et al.* (2015); de Jesus *et al.* (2015); Yang & Kim (2015)
Nuclear	EF2	West *et al.* (2008); Dixon & Saunders (2013); Hind & Saunders (2013a); Payo *et al.* (2013)
	ITS	Clarkston & Saunders (2010, 2012); Milstein & Saunders (2012); Saunders & McDevit (2012a); Preuss & Zuccarello (2014);Salomaki *et al.* (2014)
	LSU	West *et al.* (2008); Conklin *et al.* (2009); Clarkston & Saunders (2010, 2012); Clayden & Saunders (2010, 2014); Sherwood *et al.* (2010a,b); Saunders & McDevit (2012a); Manghisi *et al.* (2014); Vergés *et al.* (2014); Guoying *et al.* (2015)
	SSU	West *et al.* (2008); Peña *et al.* (2011)

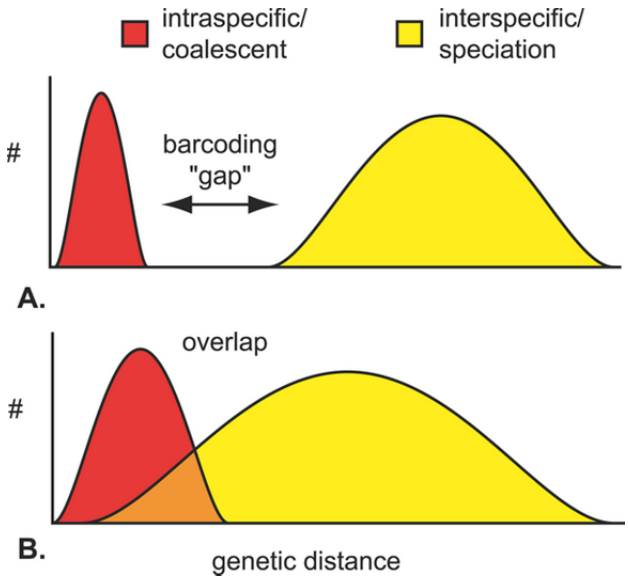


Fig. 3. "Barcode gap". The distribution of intraspecific variation is shown in red and interspecific divergence in yellow. **a**. Ideal world for barcoding, with discrete distributions and no overlap. **b**. An alternative version of the world with significant overlap and no gap. From Meyer & Paulay (2005).

To automatically identify the barcode gap in large datasets, several bioinformatics tools have been developed based on distances, standing out "Automatic Barcode Gap Discovery" (ABGD, Puillandre *et al.* 2012a) and "SPecies IDentity and Evolution in R" (SPIDER, Brown *et al.* 2012). ABGD infers the limit of intraspecific divergence and detects the barcode gap by following a recursive procedure that groups sequences into conspecific sets to get finer partitions until there is no further partition (Puillandre *et al.* 2012a). SPIDER generates two statistics for each individual in the dataset (intra- and inter-species statistics), and when the subtraction result between both statistics (intra- minus inter-specific statistic) is positive, the barcode gap is shown (see Tutorial of SPIDER software in http://spider.r-forge.r-project.org/SpiderWebSite/spider.html). Despite the growing use of DNA barcodes to delimit species (**Table 2**), these analytical tools have been rarely applied to algae. One of the few examples in seaweed is the study shown in chapter 1 in this thesis where several maerl-forming species were delimited with DNA barcodes using ABGD among other tools (published as Pardo *et al.* 2014a). Other recent examples can be found in Hernández-Kantún *et al.* (2014) and Vieira *et al.* (2014).

Likelihood methods based on evolutionary models have also been used to delimit species from an analysis of branch lengths in phylogenetic trees (Leliaert *et al.* 2014). The most popular likelihood method with a single-locus data is the General Mixed Yule Coalescent model (GMYC). GMYC determines the locations of ancestral nodes that define putative species, and

applies a likelihood ratio test to assess the fit of the branch lengths to a mixed lineage birth-population coalescence model (Pons *et al.* 2006, Fontaneto *et al.* 2007, Monaghan *et al.* 2009, Powell 2012, Fujisawa & Barraclough 2013). Like the distance methods, this approach does not rely on additional evidence to formulate hypotheses of putative species and can be applied to cases where no additional data is available to infer species limits (Fujisawa & Barraclough 2013). However, the accuracy of GMYC can be compromised when effective population sizes are high but divergence times are low (i.e. signatures of recent speciation events cannot be detected with this method; Fujisawa & Barraclough 2013). A variant of GMYC (bGMYC) implements a Bayesian methodology to account for important sources of uncertainty in the model (phylogenetic as well as in the parameters of the model) and in the specification of informative prior distributions that can increase the precision of the model (Reid & Carstens 2012). Despite the widespread use of GMYC in organisms such as reptiles (Ceccarelli *et al.* 2014), mosses (Aranda *et al.* 2014), insects (Roy *et al.* 2014) or echinoderms (Khodami *et al.* 2014), GMYC has been rarely used with marine algae (Payo *et al.* 2013, Vieira *et al.* 2014). Again, one of these few examples is shown in chapter 1 in this thesis (Pardo *et al.* 2014a), where GMYC is used as second criteria (i.e. likelihood) to delimit maerl-forming species. GMYC was also used in chapter 2 (Peña *et al.* 2015b) to describe a new major coralline forming maerl in Atlantic Iberia.

Additional methods likewise based on the concepts used by GMYC often lead to similar outputs. For example, species are identified as reciprocally monophyletic clades separated by sequence differences at least four times greater than the mean sequence difference within a clade (i.e. 4x rule) in the K/theta method. This procedure ensures that the populations from which the individuals were sampled are themselves reciprocally monophyletic with 95% probability (Birky *et al.* 2010, Birky 2013, Chen *et al.* 2014b). Recently, a simpler, faster model that should, theoretically be more robust than GMYC, was developed by Zhang *et al.* (2013). Called PTP (Poisson Tree Processes), this approach uses the number of substitutions to identify branching rate transition points instead of the time used by GMYC. Another important difference is that PTP uses a tree of standard phylogenies (i.e. non-ultrametric) as input while GMYC depends on the accuracy of a time-calibrated ultrametric input tree (i.e. trees whose tips are all equidistant from the root) to delineate species (Zhang *et al.* 2013).

Lately, single-locus approaches are being replaced by multi-locus methods under the concern that single-locus history might not be representative of organismal history (Fujita *et al.* 2012). In this context, coalescent model-based methods are gaining popularity, especially

when dealing with closely related species that are hard to tell apart with phenotypic traits (Leliaert *et al.* 2014). Several software tools have been developed in the last years to delimite species using coalescent models (Fujita *et al.* 2012). Examples are Brownie (it is named according the Brownian motion process that follows, O'Meara *et al.* 2006, O'Meara 2010) which uses a nonparametric heuristic search, and STEM (Species Tree Estimation using Maximum likelihood, Kubatko *et al.* 2009), and SpedeSTEM (SPEcies DElimitation with Species Tree Estimation using Maximum likelihood; Ence & Carstens 2011), an approach based on Maximum Likelihood (ML). Similarly, software tools based on Bayesian methods have been also developed such as BEST (Bayesian Estimation of Species Trees, Liu 2008), BEAST (Bayesian Evolutionary Analysis by Sampling Trees, Heled & Drummond 2010, Drummond *et al.* 2012), BP&P (Bayesian Phylogenetics & Phylogeography; Rannala & Yang 2003, Yang & Rannala 2014), or ABC (Approximate Bayesian Computation; Lopes & Beaumont 2010).

A Bayesian approach used in recent papers is “Bayes Factor Delimitation” (BFD), which uses the Bayes Factor (BF) as a comparison tool for model selection (Carstens & Dewey 2010, Xie *et al.* 2011, Aydin *et al.* 2014, Chen *et al.* 2014a, Grummer *et al.* 2014). BF is the ratio of the marginal likelihood of one model to the mariginal likelihood of a competing model, where the marginal likelihood measures the average fit of a model to the data (Kass & Raftery 1995, Aydin *et al.* 2014). To estimate the marginal likelihood of the competing models, several estimates can be used, as the Harmonic Mean Estimation (HME, Newton & Raftery 1994), Path Sampling (PS, Lartillot & Philippe 2006), or Steppin-stone Sampling (SS, Xie *et al.* 2011). HME often overestimates the true marginal likelihood (Xie *et al.* 2011), while PS and SS offer increased model selection accuracy compared to HME (Baele *et al.* 2012).

Regardless of the approach (single or multi-locus), it is commonly recomended that researchers should simultaneously use several methods based on different strategies to improve the confidence on their species delimitation. Concordance among methods strengthen the confidence in the resulting partition while a conservative approach should be when results are incongruent, i.e. it is preferable to fail to delimit true species than to falsely delimite entities that do not represent actual lineages, particularly when the goal of the analysis is species description (Carstens *et al.* 2013). Some authors defend that the subjectivity in delimiting taxa can be reduced by integrating multiple sources of data (e.g. molecular, morphological, ecological, and geographical). This proposal reconciles the "classical/conventional taxonomy" with the emerging molecular tools (Padial *et al.* 2010, Schlick-Steiner *et al.* 2010, Puillandre *et al.* 2012b, Carstens *et al.* 2013). Such a multi-facetted

approach has already been applied by a number of researchers (e.g. Lecocq *et al.* 2015, Wachter *et al.* 2015) is known as "integrative taxonomy" (Schlick-Steiner *et al.* 2010). In the field of seaweed, integrative taxonomy has been already successful used, especially in coralline algae (e.g. Walker *et al.* 2009, Sherwood *et al.* 2010a, Peña *et al.* 2015a). Nonetheless, when cryptic species with indistinct morphological and ecological properties are present, integrative taxonomy may not yield a clear result since the various types of data may lead to discondart conclusions (Wiens & Penkrot 2002, Fujita *et al.* 2012). In these cases, coalescent-based approaches may provide a better alternative to identify distinct lineages (Fujita *et al.* 2012). Regardless of the approach, various kinds of type material (e.g. holotype, neotype, topotype, etc.) are often included in these studies to guarantee name assignations (e.g. Hernández-Kantún *et al.* 2014, Hind *et al.* 2014a, Pardo *et al.* 2014a, Sissini *et al.* 2014, Adey *et al.* 2015, Hernández-Kantún *et al.* 2015a,b, Peña *et al.* 2015a). In several chapters of this thesis (chapters 1, 2, 3), both type material and an integrative taxonomy approach have been used to delimit/define maerl-forming species (Pardo *et al.* 2014a, Peña *et al.* 2014b, Peña *et al.* 2015b).

1.2. Assessing genetic diversity and structure with microsatellites

Once the species delimitation has been carried out, a next step to preserve its diversity involves protecting the genetic diversity of its populations. Since genetic diversity is the raw material for populations evolvuntion in response to environmental change (Frankham 1996), its loss increases the risk of population extinction (Reed & Frankham 2003, Allendorf *et al.* 2013). Genetic diversity can be measured with an array of quantitative and molecular methods/markers (Frankham *et al.* 2002, Allendorf *et al.* 2013). Among latter, typical examples are allozymes (i.e. allelic variants of enzymes), Random Amplified Polymorphic DNAs (RAPDs) markers, Restriction Fragment Length Polymorphisms (RFLPs), Amplified Fragment Length Polymorphisms (AFLPs), Sequence-Tagged Sites (STSs), microsatellites, and Single Nucleotide Polymorphisms (SNPs; Allendorf *et al.* 2013). Microsatellites, also known as Simple Sequence Repeats (SSRs) and Short Tandem Repeats (STRs), are among the most popular and suitable markers for exploring the magnitude and arrangement of genetic diversity and gene flow (i.e. connectivity) within and between algae populations (Selkoe & Toonen 2006, Arif *et al.* 2011, Krueger-Hadfield *et al.* 2011, Oppliger *et al.* 2014). They are tandem repeats of short sequence motifs 1-6 nucleotides long that occur in a high frequency in coding and non-coding regions of the nuclear genomes of most taxa (Jarne & Lagoda 1996, Goldstein & Schlotterer 1999, Selkoe & Toonen 2006), although they can also be found in chloroplastic and mitochondrial genomes

(Provan *et al.* 2001, Rajendrakumar *et al.* 2007, Kalia *et al.* 2011). Depending on motif length, they are divided into mono-, di-, tri-, tetra-, penta-, and hexanucleotides. Di-, tri- and tetranucleotide repeats are typically suitable for population genetic studies due to their high mutation rates (Li *et al.* 2002). In red algae, di- and trinucleotides often are the most abundant type (Couceiro *et al.* 2011a). On the other hand, microsatellites may be also classified according to the arrangement of nucleotides in the repeat motifs as perfect, imperfect, interrupted or composite (see **Table 3**; Oliveira *et al.* 2006, Kalia *et al.* 2011). The perfect microsatellites are more suitable to use since they have higher mutation rate than other type of microsatellites (Jin *et al.* 1996). Besides, the use of perfect microsatellites avoids homoplasy (Selkoe & Toonen 2006).

Microsatellites are highly polymorphic, even in small populations and endangered species (Allendorf *et al.* 2013). This is a consequence of a high mutation rate (10^{-2} to 10^{-6} mutations/locus/generation, Li *et al.* 2002) caused by replication slippage (**Fig. 4**), and to a recombination-like process that involves unequal cross-over and gene conversion (i.e. non reciprocal recombination) (Ellegren 2004). Other processes that generate microsatellite include the mismatch/double strand break repair (i.e. when the MisMatch Repair gene mutates or become defective, microsatellite instability increases and there is a higher rate to longer microsatellites), and retrotransposition (i.e. formation of microsatellites by retrotransposition events, e.g. microsatellites rich in A-bases in humane genome have been generated by a 3' extension of retrotranscripts, similar to mRNA polyadenylation) (Bhargava & Fuentes 2010, Kalia *et al.* 2011). Microsatellites in algae typically are less polymorphic and/or abundant than in higher plants or animals, but their variation is high enough to permit inferences of population genetic patterns (reviewed in Andreakis *et al.* 2007).

Table 3. Classification of microsatellites according to the arrangement of nucleotides in the repeat motifs.

Type of repeat sequence	Example
Perfect	ACACACACACACACACACACACACACA
Imperfect	ACACATACACTCACACGACACTACACACG
Interrupted	ACACACACAGTCTGCACACACACACACAC
Composite	ACACACACACACACTCGTCGTCGTCGTCG

Note: In a perfect microsatellite, repeat sequence is not interrupted by any base not belonging to the motif; in imperfect ones, a pair of bases between repeated motifs does not match the motif sequence. In interrupted microsatellites, a small sequence within the repetitive sequence does not match the motif sequence, while the sequence contains two adjacent distinctive sequence repeats in composite microsatellites (Oliveira *et al.* 2006, Kalia *et al.* 2011)

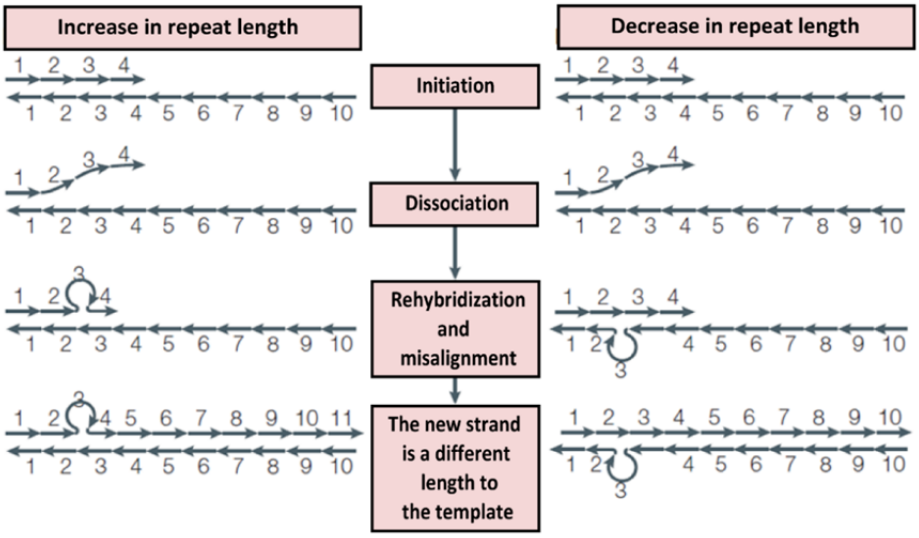


Fig. 4. Replication slippage assumes that the nascent and template strands realign out of register during DNA replication; if DNA synthesis continues unabated on this molecule, the repeat number of the microsatellite is altered. Increases in repeat length occur when misalignments introduce loops on the nascent strand. Decreases in repeat length occur when loops in the template strand lead to shorter nascent strands. Adapted from Ellegren (2004).

Thanks to their high resolution (polymorphism), codominant nature, and reliable genotyping, microsatellites are among the most widely used techniques for genome mapping, molecular ecology, and conservation studies in general (Jarne & Lagoda 1996, Goldstein & Schlotterer 1999, Selkoe & Toonen 2006, Allendorf *et al.* 2013), but they still have some drawbacks. For example, microsatellite development *de novo* requires sequence information, mutational behavior is complex, PCR artifacts (e.g. stutter bands) can occasionally be common and challenge their automated scoring, and null alleles occasionally complicate an accurate assessment of allelic/genotypic frequencies and may underestimate heterozygosity (Schlötterer 2004, Selkoe & Toonen 2006, Kumar *et al.* 2009). Nonetheless, a major drawback is that microsatellites tend to be species-specific and must be isolated *de novo* for each new species (Zane *et al.* 2002). This is due to two reasons: (i) microsatellites usually occur in non-coding regions where substitution rates are higher (Zane *et al.* 2002); (ii) nucleotide substitutions within the repeats are often observed between species when employing the same primer pair. Therefore, transferability to other species (i.e. cross-species amplification) often results in reduced levels of polymorphism even when dealing with members of the same family (Clisson *et al.* 2000, Wan *et al.* 2004). Fortunately, the Next Generation Sequencing (NGS) technologies have greatly facilitated the isolation of microsatellites on a large scale in non-model species (Schoebel *et al.* 2013), without the need of older laboratory methodologies that required cloning (Zane *et al.* 2002). Among the most popular NGS platforms (**Table 4**), 454

GS-FLX NGS technology (abbreviated as 454 NGS, see http://www.454.com/, **Fig. 5**) has been widely used for microsatellite detection (Schoebel *et al.* 2013) because it produces relatively longer reads in fast run times (**Table 4**). The probability that sequenced fragments will contain flanking regions on both sides of the microsatellite motif increases with longer reads, enabling primer design for subsequent amplification (Gardner *et al.* 2011).

NGS microsatellite projects typically generate large amounts of sequencing data from microsatellite enriched libraries (Malausa *et al.* 2011, Berthouly-Salazar *et al.* 2012, Yoshikawa *et al.* 2013, Minárik *et al.* 2014, Králová-Hromadová *et al.* 2015), which are subsequently mined for microsatellite loci. Primers are designed from regions flanking the microsatellite, and then tested to identify markers with consistent PCR amplification of unique polymorphic loci (Guichoux *et al.* 2011). High-quality PCR multiplexed reactions can be designed for these polymorphic loci primers with the help of computer programs (e.g. Multiplex Manager software develop by Holleley & Geerts 2009) to improve time and cost efficiency of the selected set of microsatellites (Guichoux *et al.* 2011).

Microsatellite development has received little attention in seaweeds in general and in Rhodophyta in particular (Andreakis *et al.* 2007). A review of the literature reveals that polymorphic, validated microsatellite markers have been reported for just 19 species of red algae from six orders (**Table 5**). Most efforts have focused on commercially valuable organisms (14 species) while non-native organisms (*Asparagopsis taxiformis* (Delile) Trevisan de Saint-Léon, *Acanthophora spicifera* (M. Vahl) Børgesen, *Gracilaria vermiculophylla* (Omhi) Papenfuss) or species of conservation concern (*Grateloupia lanceola* (J. Agardh) J. Agardh, *Pseudopolyides furcellarioides* Gallardo, Bárbara & Cremades) have received little attention (see **Table 5** for references). Couceiro *et al.* (2011a) pioneered the use of NGS technology to develop microsatellites for marine seaweeds by using 454 NGS for two non-model species of

Table 4. Comparison of the more popular NGS platforms.

Platform	Library / Temaplate preparation	NGS chemistry	Read length (bp)	Run time
454 GS-FLX*[1] (Roche)	Fragment run, Mate-pair run/Emulsion PCR	Pyrosequencing	700	23 h
HiSeq*[2] (Illumina)	Fragment run, Mate-pair run/Clonal cluster in a solid-phase	Synthesis	35	2 days
SOLiD*[3] (Life Technologies)	Fragment run, Mate-pair run/Ligation	Ligation	75	3 days for 35 bp

*[1] Specifications for 454 GS FLX Titanium System (XL+)
*[2] Older Solexa. Specifications for HiSeq 2000 System (Dual Flow Cell)
*[3] Specifications for SOLiD™ 4hq System (Fragment Library)

red algae. Compared to red algae (more of 7,000 members, Guiry & Guiry 2015), brown seaweeds had received more attention as approximately a similar number of species have validated microsatellites (20 species, see Billot *et al.* 1998, Coyer *et al.* 2002, 2009, Olsen *et al.* 2002, Whitmer 2002, Engel *et al.* 2003, 2008, Martínez *et al.* 2005, Daguin *et al.* 2006, Shi *et al.* 2007, Coleman *et al.* 2008, Collens 2009, Dolman & Coleman 2009, Faugeron *et al.* 2009, Heesch *et al.* 2010, Itou *et al.* 2012, Coelho *et al.* 2014, Bi *et al.* 2015, Zhang *et al.* 2015) have validated microsatellites despite the fact that browns form a less diverse group (more of 2,000 members, Guiry & Guiry 2015).

It is believed that microsatellites, particularly in algae, will be gradually replaced by SNPs in a near future (Putman & Carbone 2014). SNPs share many of the advantages of microsatellites (codominance, reliability) with the additional benefits of adherence to a simple infinite sites model of mutation, lack of homoplasy, and potentially thousands of loci available for analysis (Putman & Carbone 2014). Nonetheless, SNPs have not been often used in population genetic studies of non-model organisms due to a lack of data for marker ascertainment. The latter is a consequence of the procedure commonly followed for SNP generation. Most SNPs are generated by scanning genomic fragments from databases, implying that SNP discovery can produce a bias in most of the standard estimators of population genetic parameters due to an ascertainment bias in favor of common alleles (Nielsen & Signorovitch 2003, Albrechtsen *et al.* 2010, Lachance & Tishkoff 2013). In this regard, new sequencing techniques that do not require a reference genome (e.g. Restriction-site-Associated DNA seq, i.e. RAD-seq; Baird *et al.* 2008) and that use the entire sample as a discovery panel (i.e. no ascertainment bias) may help to develop and apply SNPs to population genetics studies in an immediate future as already shown in recently published studies (e.g. Hoffman *et al.* 2014, Lemay & Russello 2015, Zhai *et al.* 2015). Meanwhile, and for the first time for a coralline algae, we have developed a set of microsatellite markers using NGS technology that should serve/contribute to address issues such as genetic diversity and population structure in maerl-forming red algae (see chapter 4 and Annex I of this thesis, Pardo *et al.* 2014b, 2015b).

Table 5. Characteristics of the polymorphic microsatellite loci developed for Rhodophyta algae. Only studies that reported validated loci are included. P = perfect motif, IM = imperfect, I = interrupted, C = composite, N.A. = information not available.

Order	Species	No. (and type) of loci / allele no. / diversity (as H_E unless otherwise stated)	Reference
Bangiales	*Porphyra lines:* - *Porphyra yezoensis* Ueda (currently *Pyropia yezoensis* (Ueda) M.S. Hwang & H.G. Choi) - *Porphyra haitanensis* T.J. Chang & B.F. Zheng (currently *Pyropia haitanensis* (T.J. Chang & B.F. Zheng) N. Kikuchi & M. Miyata) - *Porphyra oligospermatangia* C.K. Tseng & B.F. Zheng - *Porphyra katadae* A. Miura (currently *Pyropia katadae* (A. Miur) M.S. Hwang, H.G. Choi, N. Kikuch & M. Miyata)	25 (23P, 2I) / 2-9/ N.A.	Sun *et al.* (2006)
	Porphyra haitanensis	11 (7P, 4I) / 3-6 / 0.182-0.955 (H_O)	Zuo *et al.* (2007)
	Porphyra haitanensis	28 (27P, 1I) / 4-15/ 0.49-0.75	Xie *et al.* (2009)
	Porphyra haitanensis	37 (P) / 2-4/ 0.206-0.289	Development by Hu *et al.* (2006). Validation by Bi *et al.* (2014)
	Porphyra yezoensis	13 (P) / N.A. / N.A.	Liu *et al.* (2005)
	Porphyra yezoensis	12 (2P, 9I, 1C) / 2-4 / 0.03-0.65	Kong *et al.* (2009)
Bonnemaisoniales	*Asparagopsis taxiformis* (Delile) Trevisan de Saint-Léon	8 (1P, 7I) / 2-7 / 0.83-0.91	Andreakis *et al.* (2007)
Ceramiales	*Acanthophora spicifera* (M. Vahl) Børgesen	5 (I) / 6-13 / 0.030-0.278 (Shannon Index)	O'Doherty & Sherwood (2007)
Gigartinales	*Chondrus crispus* Stackhouse	10 (3P, 7I) / 3-46 / 0.253-0.939	Krueger-Hadfield *et al.* (2011)
	Chondrus crispus	8 (P) / 2-12 / 0.105-0.436	Provan & Maggs (2012)
	Furcellaria lumbricalis (Hudson) J.V. Lamouroux	10 (5P, 4IM, 1I) / 8-17 (neutral loci), 4-12 (EST-derived) / 0.614-0.789 (neutral loci), 0.217-0.420 (EST-derived)	Kostamo *et al.* (2012)
	Pseudopolyides furcellarioides Gallardo, Bárbara & Cremades	5 (P) / 3-4 / 0.254-0.656	Couceiro *et al.* (2011a)

Gracilariales	*Gracilaria birdiae* Plastino & E.C. Oliveira	13 (6P, 1I, 6IM) / 2-15 / 0.059-0.854	Ayres-Ostrock *et al.* (2015)
	Gracilaria caudate J. Agardh	16 (11P, 2IM, 3I) / 2-10 / 0.059-0.810	Ayres-Ostrock *et al.* (2015)
	Gracilaria chilensis C.J. Bird, McLachlan & E.C. Oliveira	6 (1P, 4I, 1C) / 2-3 / 0.000-0.511	Guillemin *et al.* (2005)
	Gracilaria gracilis (Stackhouse) M. Steentoft, L.M. Irvine & W.F. Farnham	2 (1P, 1IM) / 10-22 / N.A.	Wattier *et al.* (1997)
	Gracilaria gracilis	9 (3P, 5I, 1C) / 2-7 / 0.000-0.640	Luo *et al.* (1999)
	Gracilaria species:	1 (P) / 5 / N.A.	Song *et al.* (2013)
	- *Gracilaria changii* (B.M. Xia & I.A. Abbott) I.A. Abbott, J. Zhang & B.M. Xia - *Gracilaria gracilis* - *Gracilariopsis lemaneiformis* (Bory de Saint-Vincent) E.Y. Dawson, Acleto & Foldvik		
	Gracilaria tenuistipitata C.F. Chang & B.M. Xia	2 (P)/ N.A. / N.A.	Song *et al.* (2014)
	Gracilaria vermiculophylla (Omhi) Papenfuss	9 (P)/ 1-5 / 0.063-0.682	Kollars *et al.* (2015)
Halymeniales	*Grateloupia lanceola* (J. Agardh) J. Agardh	7 (P) / 2-4 / 0.034-0.531	Couceiro *et al.* (2011a)
	Grateloupia filicina (J.V. Lamouroux) C. Agardh	40 (34P, 5I, 1C) / 3-10 / 0.20-0.93	Wang *et al.* (2013)

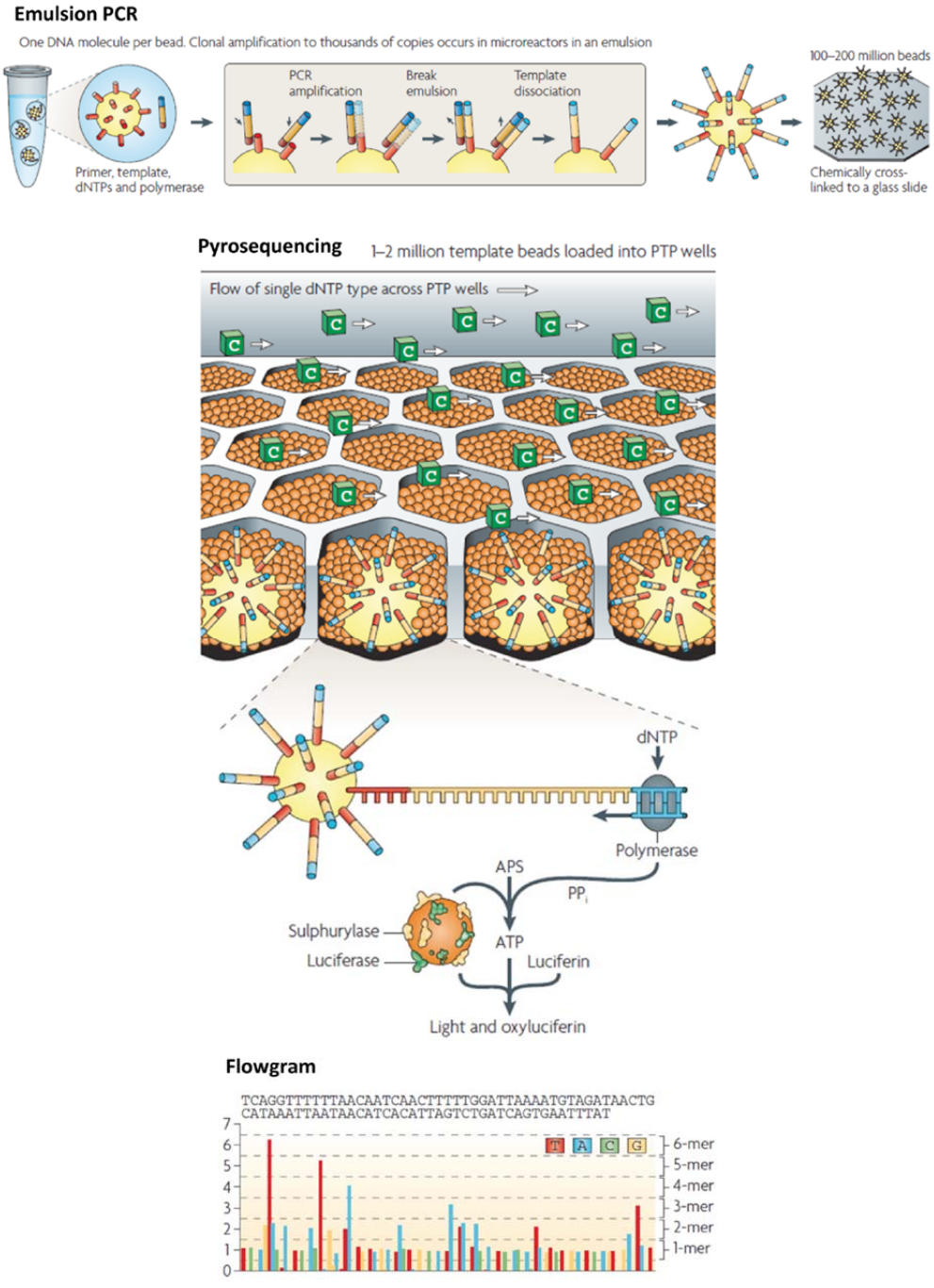


Fig. 5. 454 NGS. In emulsion PCR (emPCR), a reaction mixture consisting of an oil-aqueous emulsion is created to encapsulate bead-DNA (i.e. DNA fragments enriched in microsatellites) complexes into single aqueous droplets. PCR amplification is performed within these droplets to create beads containing several thousand copies of the same template sequence. EmPCR beads are deposited into PicoTiterPlate Wells (PTPWs). Additional beads coupled with sulphurylase and luciferase are added to individual PTPWs to perform the pyrosequencing. In this example, a single type of 2'-deoxyribonucleoside triphosphate (dNTP) –cytosine- is shown flowing across the PTPWs. The fibre-optic slide is mounted in a flow chamber, enabling the delivery of sequencing reagents to the bead-packed wells. The underneath of the fibre-optic slide is directly attached to a high-resolution Charge-Coupled Device (CCD) camera, which allows detection of the light generated from each PTPW undergoing the pyrosequencing reaction. The light generated by the enzymatic cascade is recorded as a series of peaks called a flowgram. PP_i, inorganic pyrophosphate. Adapted from Metzker (2010).

1.3. Assessing genetic diversity with microsatellites in organisms with sexual and asexual reproduction

Plants often combine sexual and asexual reproduction, especially aquatic species because water facilitates the dispersion of clones (Barrett *et al.* 1993, Silvertown 2008). The latter are individuals with identical genotype (provided that now genetic mutation takes place) generated by non-sexual mechanisms. In clonal studies, "ramets" are called to the new individuals formed by clonal spread (i.e. they are clonal units), and all ramets produced by a genotype (i.e. a single zygote) are collectively referred to a "genet" (Vallejo-Marín *et al.* 2010). The balance between sexual and asexual reproduction varies widely between and within species (Eckert 2002). Particularly in red algae, such a balance is difficult to quantify because clonal events are not restricted to asexual reproduction. In red seaweed (see section 3 below), sexual reproduction produces a zygote that gives rise to a diploid carpospores-producing plant. This plant produces identical diploid carspospores (i.e. "clonal carpospores") derived from a single zygote. When carpospores are released, they develop into diploid tetrasporangial thalli with identical genotype. Identical genotypes are likewise generated in asexual reproduction events (no recombination) such as the fragmentation of sporangial plants (Woelkerling 1988). Therefore, if the production of carpospores should be considered a mechanism for clones generation, genetically identical individuals in red algae populations (somatic mutations provided) are difficult to assign to sexual or asexual reproduction events.

The rate of sexual *vs*. asexual reproduction in natural populations has a crucial influence on demography and genetics (Eckert 2002, Halkett *et al.* 2005, Vallejo-Marín *et al.* 2010). The composition and evolution of populations of clonal plants are largely affected by the level of intraspecific competition (i.e. turnover of genets and intensity of inter-genet competition for space; Eriksson 1993, Pan & Price 2001, Travis & Hester 2005), and the fittest genotype can rapidly spread as a clonal lineage over the entire population, rapidly colonizing the habitat (Eriksson 1993, Arnaud-Haond *et al.* 2010, Becheler *et al.* 2010, Becheler *et al.* 2014a,b). Nonetheless, in habitats where sexual reproduction is prevented, either because of the absence of mating partners or because ecological conditions are unfavorable to spores, asexual spread may be the only type of reproduction and, consequently, survival (Eckert & Barrett 1993, Honnay & Bossuyt 2005, Silvertown 2008). Alternatively, extensive clonality can have broad fitness costs as a result of inbreeding depression (Balloux *et al.* 2003), and clonal populations may be poorly equipped to adapt to environmental change (Balloux *et al.* 2003, Honnay & Bossuyt 2005, Silvertown 2008).

Genetic inferences on the rate of clonal reproduction require rigorous analyses that must be based on an adequate sampling strategy to ensure that sample size is as big as possible but, also, that there is no hidden genetic structuring within the units defined as sub-populations to avoid a Wahlund effect (Halkett *et al.* 2005). The latter is known to strongly influence parameter estimates such as *F*-statistics and linkage disequilibrium (*LD*), and tends to mimic the signal of clonal reproduction (Halkett *et al.* 2005, Arnaud-Haond *et al.* 2007). Co-dominant markers are preferable when working with diploid or polyploidy species because several test of clonal reproduction depend on the amount of heterozygosity, and microsatellites are often considered the markers of choice (Halkett *et al.* 2005, Guillemin *et al.* 2008, Yakimowski & Barrett 2014). Additionally, a good genotypic resolution is required when using microsatellites for the quantitative and qualitative assessment of genetic diversity in populations of clonal organisms (Arnaud-Haond *et al.* 2005, Millar *et al.* 2010). Methods to assess clonality in population studies are still at their infancy, but a number of steps are often followed when searching for clonality (**Fig. 6**; Halkett *et al.* 2005). Once the genetic data has been collected, the index of clonal diversity (*R*) proposed by Dorken & Eckert (2001) must be estimated because the presence of repeated MultiLocus Genotypes (MLGs) is the most obvious signal of clonal spread in a population (**Fig. 6**):

$$R = \frac{G - 1}{N - 1}$$

where *G* is the number of MLGs detected in the population (i.e. genotypic richness), and *N* is the number of individuals sampled. *R*=0 indicates a strictly clonal population where all individuals share the same genotype, whereas *R*=1 suggests that the population must exclusively reproduce by a sexual strategy (Halkett *et al.* 2005, Arnaud-Haond *et al.* 2007). In this context, the occurrence of repeated MLGs can be regarded as a strong evidence of clonal reproduction, although it cannot be taken as a definitive proof (Halkett *et al.* 2005).

The next step for visualizing clonal spread is to detect non-random associations between loci mimicking complete physical linkage over the entire genome. Consequently, *LD* should be estimated (**Fig. 6**; Halkett *et al.* 2005). Finally, the apportionment of genetic variance between the individual and the population levels is the last estimate to prove the occurrence of clonal reproduction. Under clonal spread, F_{IS} (i.e. the deviation from random mating within subpopulations) is expected to be negative, indicating an excess of heterozygotes relative to random mating (**Fig. 6**; Halkett *et al.* 2005).

Once the clonal signal has been confirmed, clonal diversity and structure should be quantified. The latter can be performed with the help of specific software such as GENCLONE (Arnaud-Haond & Belkhir 2007). Clonal diversity analyses with this software involve a first stage of clonal discrimination where the likelihood that two individuals with the same MLG are indeed part of the same clone rather than the result from distinct sexual reproductive events (i.e. distinct clonal lineages: Multilocus Lineage or MLLs) is estimated. The probability for a given MLG to be observed in *N* samples as a consequence of different sexual reproductive events (*Psex*, see Box 1 in Arnaud-Haond *et al.* 2007) is calculated for each repetated MLG. If *Psex* > 0.05, duplicated MLGs are considered as different genets or different MLLs. If *Psex* is < 0.05, duplicated MLGs are considered clones of the same genetic individual (i.e. genet) or the same MLL (Arnaud-Haond *et al.* 2007).

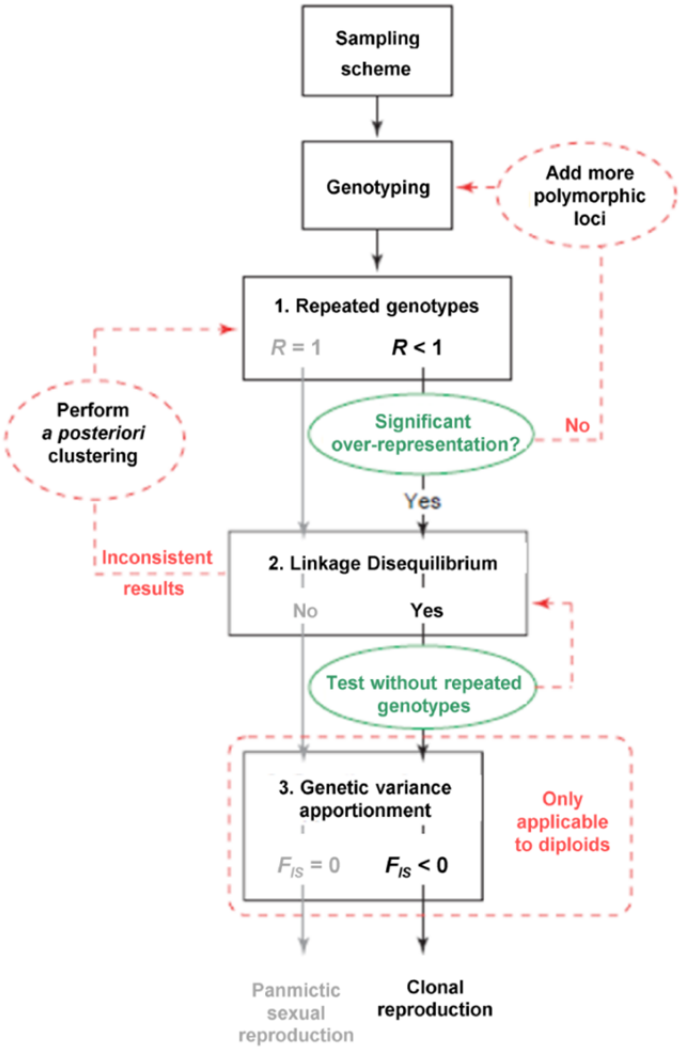


Fig. 6. Sequential inference of population clonal rate. Modified from Halkett *et al.* (2005).

Clonal heterogeneity, i.e. the distribution of the sampling units among MLLs (Arnaud-Haond *et al.* 2007), is a fundamental feature that determines the ecology and evolution of populations. The index most commonly used to calculate this feature is based on the famous Simpson index:

$$D^* = 1 - \sum_{i=1}^{G} p_i^2;$$

where *G* is the number of MLLs detected in the sample, and p_i is the frequency of the *i*th MLL in the population. Unbiased estimates of p_i are given by:

$$p_i^2 = \left[\frac{n_i(n_i - 1)}{N(N-1)}\right]$$

where *N* is the number of individuals sampled and n_i is the number of sample units sharing the *i*th MLL. This index can be interpreted as an estimate of the probability that two random individuals from the population would belong to the same MLL (Arnaud-Haond *et al.* 2007). As the index of heterogeneity does not reflect equitability, some estimate of clonal evenness must be obtained. Again, a version of the widely used evenness index of Simpson can be used:

$$ED^* = \frac{(D - D_{min})}{(D_{max} - D_{min})}$$

where D_{min} and D_{max} are the approximate minimum and maximum values of adapted Simpson's index for genotypic diversity given a sample size *N* and a sample genotypic richness *G*:

$$D_{min} = \frac{(2N-G)x(G-1)}{N^2} x \frac{N}{N-1} \quad \text{and} \quad D_{max} = \frac{(G-1)}{G} x \frac{N}{N-1}$$

The Simpson's evenness index varies from 0 when only one MLG is observed, to 1 when all MLLs have equal abundance (Arnaud-Haond *et al.* 2007).

The clonal distribution of ramets into classes (MLLs, or genets) is assessed with a density distribution (Arnaud-Haond *et al.* 2007). The Pareto distribution is often used in clonal studies; it describes a skewed distribution of genotypes among lineages (Arnaud-Haond *et al.* 2010, Becheler *et al.* 2010, 2014a,b). The parameter of this distribution β (derived from the slope) and the maximum MLLs size (in terms of number of size replicates) are used as indicators of

evenness and diversity, providing an intuitive graphical depiction of the heterogeneity in the distribution of replicates among lineages (Arnaud-Haond *et al.* 2007).

Two descriptors are often used for clonal structure analysis: clonal subrange and aggregation of clonal lineages (Millar *et al.* 2010, Becheler *et al.* 2014a, Binks *et al.* 2015). Clonal Subrange (CS, as clonal identity in Harada *et al.* 1997) is calculated as the characteristic maximum size of MLLs in the sample, and the spatial scale beyond which clonality does not affect genetic structure (Arnaud-Haond *et al.* 2007). The aggregation of the MLLs is estimated with the spatial aggregation index:

$$A_c = \frac{(P_{sg} - P_{sp})}{P_{sg}}$$

where P_{sg} is the average probability of clonal identity of all sample unit pairs, and P_{sp} is the average probability of clonal identity among pairwise nearest neighbours (Arnaud-Haond *et al.* 2007). The aggregation index varies between 0 (high intermingling) to 1 (high spatial aggregation of ramets). Significance is tested against the null hypothesis of a spatially random distribution of identical MLGs using a resampling approach where the individuals are randomly assigned to the existing sampling coordinates (Arnaud-Haond *et al.* 2007, Millar *et al.* 2010).

2. Maerl beds in Atlantic Europe

"Maerl beds" are accumulations of slow-growing, unattached non-geniculate coralline red algae that create biogenic habitats with a high biodiversity (**Fig. 7**). They are considered hotspots of marine life (Foster 2001, Nelson 2009, Hall-Spencer *et al.* 2010, Peña *et al.* 2014a). The high abundance and biodiversity of associated species in comparison to surrounding habitats is generally attributed to the three-dimensional structure of maerl deposits that provides a wide range of niches. Maerl beds also act as nursery areas for different organisms, many of them with commercial value (BIOMAERL Team 2003, Kamenos *et al.* 2004a-d).

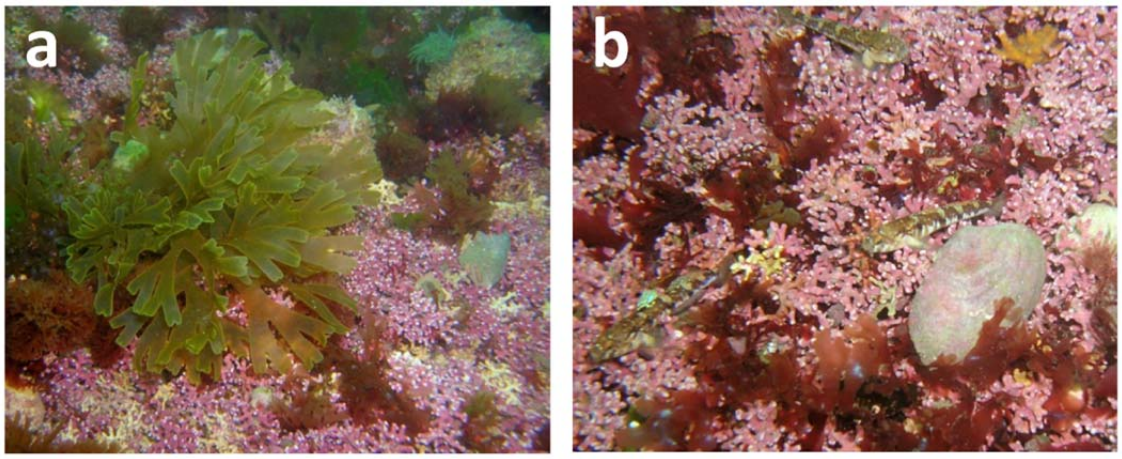


Fig. 7. Maerl bed in Benencia (Ría de Arousa, Galicia, NW Spain). **a**. General view. **b**. Detailed view.

2.1. Distribution, threats and protection status

Maerl beds occur from the poles to the tropics forming extensive communities (Foster 2001, Nelson 2009). They are regarded as one of the Earth's "Big Four" benthic communities dominated by marine macrophytes (together with kelp beds and forest, seagrass meadows, and non-geniculate coralline reefs; Foster 2001). In Atlantic Europe, there are maerl habitats from Svalbard to south Portugal (**Fig.8**), as well as in Macaronesia, in a wide variety of hydrodynamic conditions, from lower intertidal to 60 m depth (Grall 2003, Hall-Spencer *et al.* 2010, Peña Freire 2010, Peña *et al.* 2014a). However, most of the studies conducted so far have focused on beds from the British Isles (mostly Scotland and Ireland), French Brittany, and Galicia (NW Spain) (Grall 2003, Peña & Bárbara 2009, Hall-Spencer *et al.* 2010).

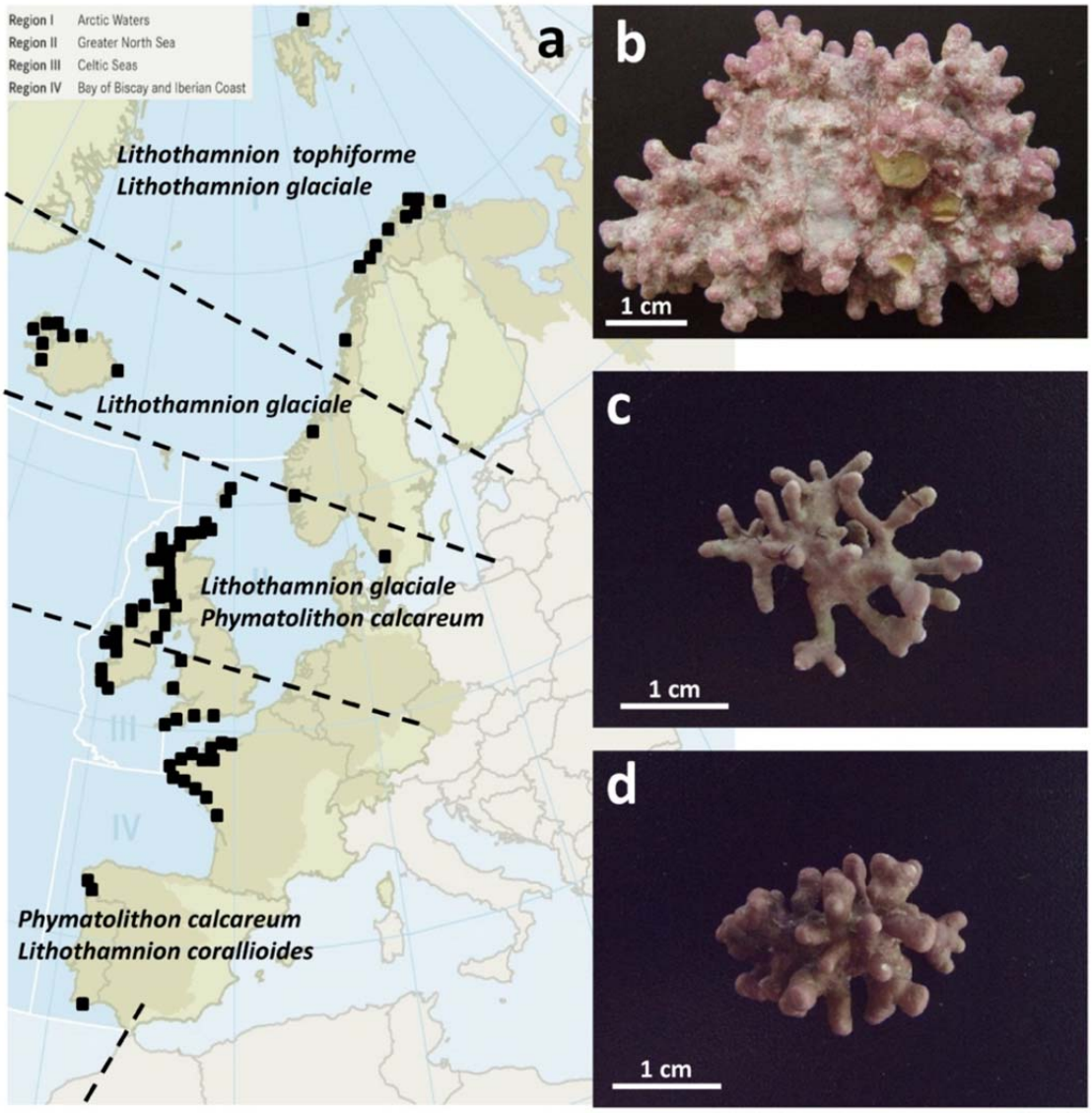


Fig. 8. **a**. Distribution of maerl beds, and major maerl-forming species in Atlantic Europe conventionally reported before our studies. Data from Grall (2003) and Peña *et al.* (2014a). **b**. *Lithothamnion glaciale* (voucher CPVP-1443, Svalbard, Norway). **c**. *Phymatolithon calcareum* (voucher CPVP-654, Galicia, NW Spain). **d**. *Lithothamnion corallioides* (voucher CPVP-816, Brittany, NW France).

Maerl beds have been exploited as soil amendments and for other commercial purposes, particularly in Brittany and the British Isles (Briand 1991, Birkett *et al.* 1998, Grall 2003, Grall & Hall-Spencer 2003). Maerl extraction causes substratum loss and abrasion; additionally, dredging generates turbidity that reduces light penetration and also smothers live-maerl (Jones *et al.* 2000). Others human threats to maerl are fishing (bottom fisheries), aquaculture (e.g. mussel rafts and fish cages), recreation (mooring chains and anchors), invasive species (e.g. *Crepidula fornicata* L. 1758), civil works (breakwaters, quays, sea-walls, yacht marinas), and even eutrophication (from domestic and/or sewage sludge and industrial

waste discharged at sea through long pipelines, dumping of harbour dredged sediments) (BIOMAERL Team 2003, Peña & Bárbara 2008a, Hall-Spencer *et al.* 2010, Peña Freire 2010). These threats can negatively impact the conservation status and structure of maerl beds (**Table 6**). More recently, climate change/global warming and ocean acidification have been identified as potentially important threats for maerl beds in a near future (Nelson 2009, Noisette *et al.* 2013a,b, Brodie *et al.* 2014, McCoy & Kamenos 2015). Growth studies with coralline algae under acidic conditions show a negative relationship among both parameters (e.g. Jokiel *et al.* 2008). The negative impact of acidification on coralline calcificaton may be exacerbated by ocean warming (Anthony *et al.* 2008). In the particular case of maerl-forming species, it has been anticipated that corrosion of their high Mg-calcite skeletons will systematically occur with the ocean acidification and global warming; as consequence, the dissolution of dead and, to a lesser extent, live maerl habitat in areas of the northeast Atlantic is expected (Nelson 2009, Büdenbender *et al.* 2011, Brodie *et al.* 2014). On the other hand, maerl skeletons may lose their structural integrity under high CO_2 conditions, facilitating the release of DMSP (a secondary metabolite detected in high concentrations in temperate maerl-forming species that combats oxidative stress; Rix *et al.* 2012) to the water column. High concentrarion of DMSP in the water column can stimulate the microbial production of DMS, altering the sulfur cycle (Burdett *et al.* 2012, Brodie *et al.* 2014, McCoy & Kamenos 2015).

The negative impacts of these threats explain that maerl beds have declined in both extent and quality along the European coasts since the las century (BIOMAERL Team 2003, Peña & Bárbara 2008a, Hall-Spencer *et al.* 2010). Legislative action to preserve them has been notably timid. Maerl beds are indirectly included in two specific marine habitats listed under

Table 6. Negative impacts on maerl beds. Extracted from BIOMAERL Team (2003), Hall-Spencer *et al.* (2010) and Peña Freire (2010).

Type of impact	Impacts
Physical	Reduction of light penetration due to sediment resuspension Alteration of inshore currents Burial of live maerl Granulometric changes
Chemical	Increase in organic matter Changes in the geochemistry of the seabed Eutrophication
Biological	Decrease of habitat complexity (i.e. decrease in species richness and abundance) Changes in species composition Substitution of indigenous biota by invasive species

Annex I of the Habitats Directive ("sandback covered by seawater all the time" and "large shallow inlets and bays"). In addition, the two major maerl-forming species reported in the literature, *Phymatolithon calcareum* (Pallas) W.H. Adey & D.L. McKibbin and *Lithothamnion corallioides* (P.L. Crouan & H.M. Crouan) P.L. Crouan & H.M. Crouan, have been listed in Annex V as species whose exploitation must be compatible with maintaining a favourable conservation status. Maerl has been also defined in the European Nature Information System (EUNIS) under different habitats, and it has been an important feature of Natura 2000 sites. In addition, maerl beds have been also listed in the OSPAR (The Convention for the Protection of the Marine Environment of the North-East Atlantic) List of Threatened and/or Declining Species and Habitats (Hall-Spencer *et al.* 2010).

2.2. Diversity of maerl-forming species

According to the literature, maerl-forming species of Atlantic Europe belong to six genera from Corallinales and Hapalidiales (Cabioch 1974, Irvine & Chamberlain 1994, Le Gall & Saunders 2007, Rosas-Alquicira *et al.* 2009, Nelson *et al.* 2015), as shown below:

Phylum Rhodophyta
Class Florideophyceae
Subclass Corallinophycidae
Order Corallinales
Family Corallinaceae
Subfamily Lithophylloideae
Genus *Lithophyllum*
Subfamily Mastophorideae
Genus *Spongites*
Subfamily Neogoniolithoideae
Genus *Neogonioliton*
Order Hapalidiales
Family Hapalidiaceae
Subfamily Melobesioideae
Genus *Lithothamnion*
Genus *Mesophyllum*
Genus *Phymatolithon*

Before the publication of chapter 1 of this thesis, and based on the number of records, major maerl-forming species cited in the literature for Atlantic Europe were *Phymatolithon calcareum* and *Lithothamnion corallioides*, together with *L. tophiforme,* and *L. glaciale* (Hall-Spencer *et al.* 2010, **Fig. 8**, **Table 7**). Other minor maerl-forming species were also reported in the literature. Some of them had been described/reported in the 19th century and early 20th, but went unnoticed since then: *Lithothamnion fruticulosum*, *L. breviaxe*, *L. fornicatum*, *L.*

nodulosum, *L. tusterense*, *L. intermedium*, *L. ungeri*, and *L. norvegicum* (for references see **Table 7**).

Until 2011, the identification of the maerl-forming species compiled in **Table 7** relied on conventional taxonomic practices (i.e. morphological/anatomical identification). However, later studies with molecular markers have shown that the coralline algae may hide considerable cryptic diversity due to their high phenotypic plasticity and lack of diagnostic features (e.g. Robba *et al.* 2006, Walker *et al.* 2009, Pardo *et al.* 2015a). With the suspicion that under maerl-forming corallines may also hide considerable cryptic diversity, a multi-loci molecular assessment of the species present in maerl beds of Atlantic Europe was the starting point of this thesis. Results confirmed our suspiction because a new major species was found widespread in Iberia, *Phymatolithon* sp.3 (Carro *et al.* 2014, Pardo *et al.* 2014a), recently re-named as *Phymatolithon lusitanicum* V. Peña. Details on these results can be seen in chapters 1 and 2 of this thesis.

Table 7. Maerl-forming species reported in Atlantic Europe and Macaronesia prior to our results.

	Atlantic Europe											Macaronesia		
	Svalbard Archipelago	**Greenland**	**Iceland**	**Scandinavia**	**Scotland**	**Britain**	**Ireland**	**Brittany**	**Bay of Biscay**	**Galicia**	**Portugal**	**Azores**	**Madeira**	**Canary Islands**
Major maerl-forming species														
Lithothamnion tophiforme (Esper) Unger	Hall-Spencer *et al.* (2010)	Unger (1858) Adey *et al.* (2005) Hall-Spencer *et al.* (2010)	Strömfelt (1886) Adey (1971) Gunnarsson (1977)	Foslie (1895) Adey (1971) Hall-Spencer *et al.* (2010)	-	-	-	-	-	-	-	-	-	-
Lithothamnion glaciale Kjellman	Kjellman (1883) Teichert *et al.* (2012)	Rosenvinge (1894) Foslie (1905) Hall-Spencer *et al.* (2010)	Foslie (1905) Adey (1971) Gunnarsson (1977)	Foslie (1895) Foslie (1905) Adey (1971) Hall-Spencer *et al.* (2010)	Foslie (1905) Adey & Adey (1973) Irvine & Chamberlain (1994) Hall-Spencer *et al.* (2010)	Adey & Adey (1973) Irvine & Chamberlain (1994) Hall-Spencer *et al.* (2010)	Adey & Adey (1973) Irvine & Chamberlain (1994)	-	-	-	-	-	-	-
Phyamtolithon calcareum (Pallas) W.H. Adey & D.L. McKibbin	-	-	-	Suneson (1958) Adey (1971) Hall-Spencer *et al.* (2010)	Hall-Spencer & Moore (2000)	Adey & Adey (1973) Woelkerling & Irvine (1986) Irvine & Chamberlain (1994)	Adey & Adey (1973) Irvine & Chamberlain (1994)	Lemoine (1910) Mendoza & Cabioch (1998) Hall-Spencer *et al.* (2010)	Sauriau *et al.* (2012)	Adey & McKibbin (1970) Peña & Bárbara (2004, 2008b)	Peña *et al.* (2009)	Rosas-Alquicira *et al.* (2009)	-	-
Lithothamnion corallioides (P.L.Crouan & H.M.Crouan) P.L.Crouan & H.M.Crouan	-	-	-	Foslie (1895)	-	Foslie (1895) Irvine & Chamberlain (1994) Hall-Spencer *et al.* (2010)	Adey & Adey (1973) Irvine & Chamberlain (1994) Hall-Spencer *et al.* (2010)	Cabioch (1969) Mendoza & Cabioch (1998) Hall-Spencer *et al.* (2010)	-	Adey & McKibbin (1970) Peña & Bárbara (2004)	-	-	Cabioch (1974)	Afonso-Carrillo & Gil-Rodríguez (1982) Hall-Spencer *et al.* (2010)

Minor maerl-forming species (narrow or uncertain distribution)														
Lithothamnion fruticulosum (Kützing) Foslie	-	Foslie (1895)	Strömfelt (1886)	Foslie (1895)	-	Foslie (1895)	Guiry (1978)	-	-	-	-	-	-	-
Lithothamnion breviaxe Foslie	-	-	-	Foslie (1895)	-	-	-	-	-	-	-	-	-	-
Lithothamnion fornicatum Foslie	-	-	-	Foslie (1895)	-	-	-	-	-	-	-	-	-	-
Lithothamnion intermedium Kjellman	-	-	-	Kjellman (1883) Foslie & Printz (1929)	-	-	-	-	-	-	-	-	-	-
Lithothamnion nodulosum Foslie	-	-	-	Foslie (1895)	-	-	-	-	-	-	-	-	-	-
Lithothamnion tusterense Foslie	-	-	-	Foslie & Printz (1929)	-	-	-	-	-	-	-	-	-	-
Lithothamnion ungeri Kjellman	-	-	-	Kjellman (1883) Foslie (1895)	-	-	-	-	-	-	-	-	-	-
Lithothamnion norvegicum (Areschoug) Kjellman	-	-	-	Kjellman (1883) Foslie & Printz (1929)	Foslie & Printz (1929)	-	-	-	-	-	-	-	-	-
Lithothamnion lemoineae Adey	-	-	-	-	Irvine & Chamberlain (1994) Hall-Spencer *et al.* (2010)	-	-	-	-	-	-	-	-	-

Lithothamnion sonderi Hauck	-	-	-	-	Hall-Spencer *et al.* (2010)	-	-	-	-	-	-	-	-	-
Phymatolithon purpureum (P.L. Crouan & H.M. Crouan) Woelkerling & L.M. Irvine	-	-	-	Adey & Adey (1973)	-	-	Adey & Adey (1973) Irvine & Chamberlain (1994)	-	-	-	-	-	-	-
Lithophyllum duckerae Woelkerling	-	-	-	-	-	Irvine & Chamberlain (1994)	-	-	-	-	-	-	-	-
Litthophyllum hibernicum Foslie	-	-	-	-	-	-	Foslie (1906) Irvine & Chamberlain (1994) Hall-Spencer *et al.* (2010)	-	-	-	-	-	-	-
Mesophyllum lichenoides (J. Ellis) Me. Lemoine	-	-	-	-	-	-	Lemoine (1913)	-	-	-	-	-	-	-
Lithophyllum fasciculatum (Lamarck) Foslie	-	-	-	-	-	Irvine & Chamberlain (1994) Hall-Spencer *et al.* (2010)	Foslie (1899) Irvine & Chamberlain (1994) Hall-Spencer *et al.* (2010)	Cabioch (1969) Hall-Spencer *et al.* (2010)	-	-	-	-	-	-
Lithophyllum dentatum (Kützing) Foslie	-	-	-	-	-	-	Foslie (1900) Irvine & Chamberlain (1994) Hall-Spencer *et al.* (2010)	Hall-Spencer *et al.* (2010)	-	-	-	-	-	-

Mesophyllum sphaericum V. Peña, Bárbara, W.H. Adey, Riosmena-Rodríguez & H.G. Choi	-	-	-	-	-	-	-	-	-	Peña *et al.* (2011)	-	-	-	-
Neogoniolithon brassica-florida (Harvey) Setchell & L.R. Mason	-	-	-	-	-	-	-	-	-	-	-	Rosas-Alquicira *et al.* (2009)	-	-
Lithophyllum crouanii Foslie	-	-	-	-	-	-	-	-	-	-	-	Rosas-Alquicira *et al.* (2009)	-	-
Spongites fruticulosa Kützing	-	-	-	-	-	-	-	-	-	-	-	-	Cabioch (1974)	-

Records restricted to reports as maerl; some species have wider reported ranges as encrusting forms. Currently accepted names are used except for *Lithothamnion fruticulosum* where an older name is retained (see further details in Woelkerling 1985).

3. Life-history in non-geniculate coralline red algae

Non-geniculate coralline red algae combine both sexual and asexual reproduction (Johansen 1981, Woelkerling 1988). The life cycle involves three phases (**Fig. 9**): a haploid gamete-producing phase (i.e. gametophyte, n), a diploid carpospores-producing phase (i.e. carposporophyte, 2n), and a diploid tetraspore-producing phase (i.e. tetrasporophyte, 2n). Except for Sporolithales, both gametes and spores are produced within conceptacles. Generally, male and female conceptacles are formed on separate plants. Conceptacles with male gametes, female gametes and carpospores are uniporates, i.e. a single pore at the roof through which gametes or spores pass (**Fig. 9**). Depending on the order, conceptacles with tetraspores (or bispores) are either uniporate (Corallinales) or multiporate (Hapalidiales) (**Fig. 9**). Male gametes (spermatia) released from the conceptacle travel to the female conceptacles and fertilise the female gametes when plasmogamy (gamete fusion) and karyogamy (fusion of male and female nuclei) take place. The resulting zygote undergoes a complex series of changes that ultimately result in the production of a microscopic, diploid carpospores-producing plant within the female conceptacle. Carposporophytes usually consist of a central fusion cell, and unbranched filaments (gonimoblast filaments) each one bearing a terminal carposporangium with one carpospore. Diploid carpospores produced by a single carposporophyte are genetically identical. After release, they develop into independent, diploid, tetraspore-producing plants that form haploid tetraspores by meiosis. Mature tetraspores released from the conceptacles germinate and develop into new gametophytes, thereby completing the sexual cycle (Woelkerling 1988).

Asexual reproduction may occur in several ways (**Fig. 9**). Bisporangia seem relatively common in corallines (Maggs 1988), and they may form within uniporate or multiporate conceptacles on tetrasporangial (2n) and bisporangial (2n) plants (Woelkerling 1988, Guiry 1990, Irvine & Chamberlain 1994). In general, meiosis is supposed to precede the formation of binucleate bispores (n) that are considered to give rise to sexual plants, i.e. gamethophytes (Suneson 1950, **Fig. 9**). However, uninucleate bispores (2n) produced by apomeiotic division (Suneson 1950, 1982) have been observed to be commoner (Bauch 1937), and seemingly self-perpetuate the plant without sexual reproduction (**Fig. 9**), especially in Boreal-Arctic and Antarctic regions (Suneson 1950, 1982). On the other hand, aberrant events sometimes occur such as the production of bisporangia mixed with tetrasporangia within the same conceptacle (Suneson 1937, Edyvean & Ford 1986); both structures show uninucleate spores (Suneson

1937). Another aberrations are conceptacles with bisporangia and carpogonia mixed (Suneson 1943), pentasporangia mixed with tetrasporangia and bisporangia (Woelkerling & Campbell 1992), tetrasporangia with three ingrown cleavage septa with only two nuclei (Suneson 1950), bisporangia with four nuclei (Suneson 1950), and even trisporangia (Bauch 1937, Woelkerling & Campbell 1992). The latter was found, for example, in *Phymatolithon* (as *Lithothamnion* in Suneson 1943).

Thallus fragmentation is another alternative for asexual spread in non-geniculate corallines. Fragmentation seems a common process in these algae, where a broken thallus keeps growing to form a new individual (**Fig. 9**, Johansen 1981, Woelkerling 1988). This strategy may be particularly efficient at establishing new locations, as seen in other red algae (Herren *et al.* 2013). Finally, propagule production is another type of asexual reproduction in non-geniculatate coralline algae (**Fig. 9**, Johansen 1981). Propagules are groups of vegetative cells that form a structure with distinctive morphology that detaches from the parent thallus and gives rise to a new individual (Dolms-Lasubach 1881). One example is *Hydrolithon farinosum* (J. V. Lamouroux) Penrose & Y. M. Chamberlain, where propagules were studied by Coppejans (1978, under the name *Fosliella farinosa* (J.V. Lamouroux) M. Howe). With exception of this species, reproduction by propagules is a poorly known phenomenon in Rhodophyta in general. Nonetheless, propagule production may contribute to population increase, to weather unpredictable environmental changes, to survive in conditions that would be lethal for entire thalli, and to reach new habitats (Cecere *et al.* 2011).

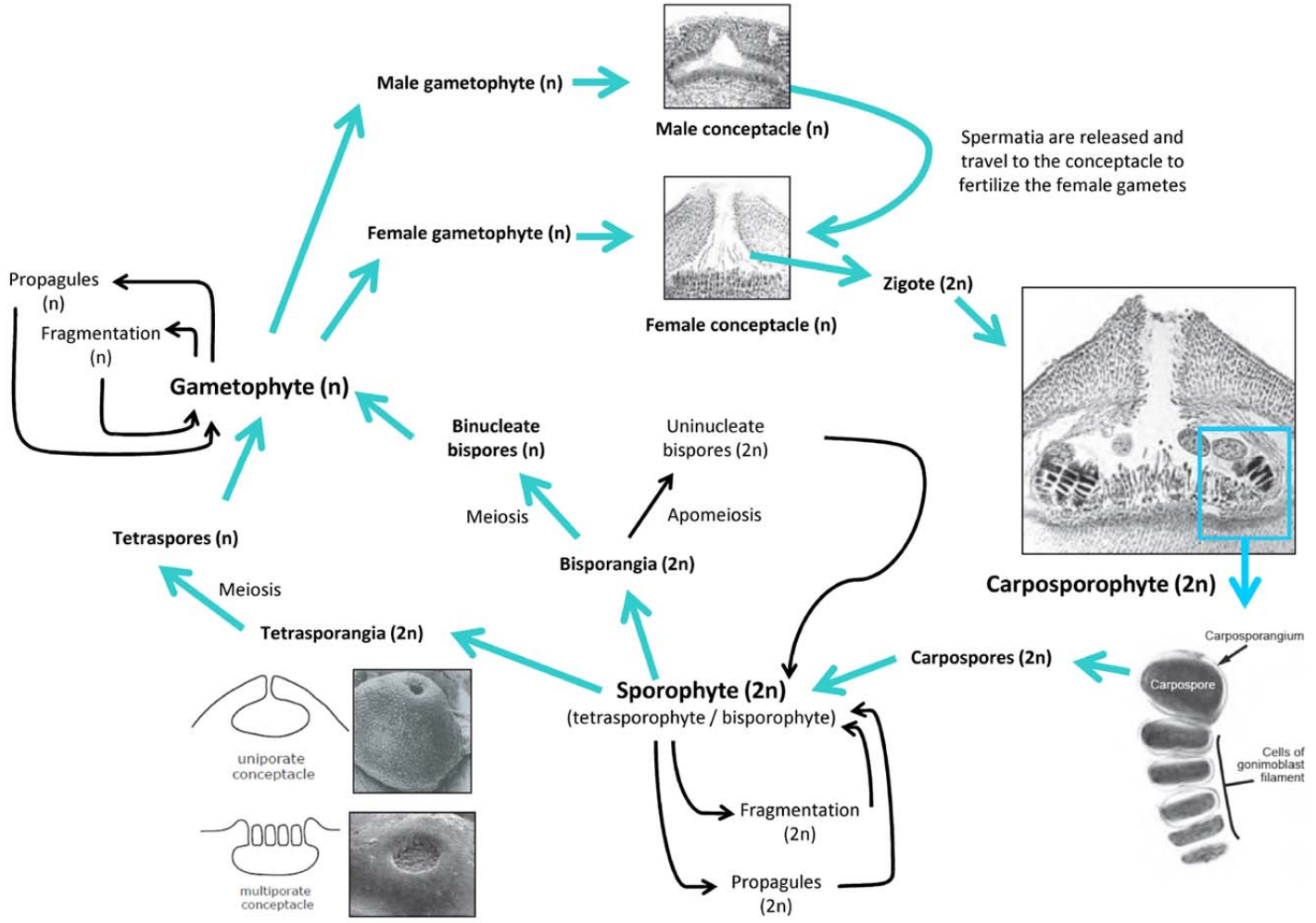


Fig. 9. Life history in non-geniculate coralline red algae. Blue arrows: sexual cycle. Black arrows: asexual cycle. Adapted from Woelkerling (1988). Photos and pictures from Harvey *et al.* (2005) and Farr *et al.* (2009), respectively..

3.1. Life-history in maerl-forming species

Thallus fragmentation has often been suggested as the main method of reproduction in maerl-forming species (**Fig. 10**, Bosence 1976, Johansen 1981), possibly because most of the studies on the taxonomy and ecology of maerl-forming species noted that reproductive structures are rarely found (Birkett *et al.* 1998). For example, no fertile thallus of *Lithothamnion corallioides* was detected in a two-year-long monthly sampling survey of maerl beds in Galway Bay (Maggs 1983). Similarly, Cabioch (1970) reported that fertile structures in maerl-forming species occur only every five or six years in maerl beds from Brittany. Understandably, only occasional reports of multiporate sporangial conceptacles exist for *P. calcareum* and *L. corallioides* in Atlantic Europe (**Fig. 10**, Suneson 1958, Adey & McKibbin 1970, Woelkerling & Irvine 1986, Irvine & Chamberlain 1994, Peña & Bárbara 2004, 2008b). Also, aberrant reproductive events were seen in maerl-forming species: bisporangia were occasionally seen intermingled with tetrasporangia in conceptacles of *P. calcareum* from Scandinavia (as *Lithothamnion* in Suneson 1958).

Fertile gametophytes seem even rarer in Atlantic Europe, and they have been exclusively detected in *Phymatolithon calcareum* and *Lithothamnion corallioides* as crusts (i.e. attached growth form) growing on pebbles, gravel and dead maerl in a subtidal bed from Brittany (Cabioch 1969, 1970, Mendoza & Cabioch 1998). Additionally, Cabioch (1970) suggested that the sexual cycle in maerl-forming species occurs each 5-6 years in Brittany maerl beds. Despite the shortage of records for the sexual phase, the life history of *P. calcareum* and *L. corallioides* depicted by Bosence (1976) on the basis of studies conducted by Adey & McKibbin (1970) and Cabioch (1972), suggests that the recruitment of new plants (i.e. unattached growth form) in maerl beds is produced by the break off of erect branches derived from fecunded gamethophytes (i.e. crust, **Fig. 10**).

The information on life history of maerl-forming species compiled from the literature is based on morphological and anatomical studies (Adey & McKibbin 1970, Cabioch 1970, 1972, Bosence 1976, Mendoza & Cabioch 1998). The molecular tools currently available have opened the war to more reliable studies on the reproduction of maerl-forming species, especially the gametophytic phase. In this thesis, a confirmation of identity of the crustose gametophytic phase of *P. calcareum* was performed using DNA barcodes as molecular tool (see chapter 3, Peña *et al.* 2014b).

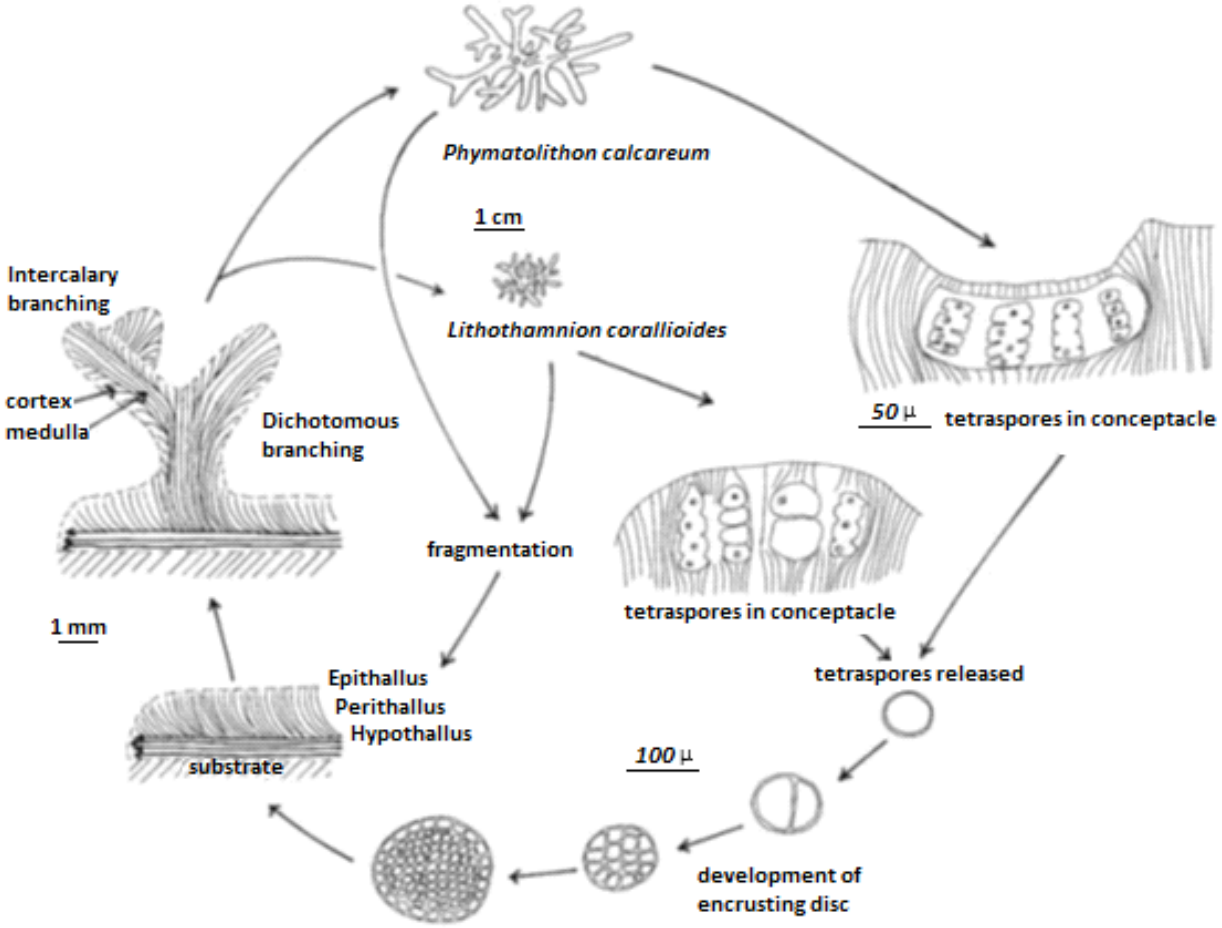


Fig. 10. Life history of *Phymatolithon calcareum* and *Lithothamnion corallioides*. Adapted from Bosence (1976).

4. Aims of this thesis

The previous sections of this introduction describe some of the gaps in our knowledge of maerl beds in Atlantic Europe. Key details on species diversity, life cycle, and population genetics of maerl-forming species are still incompletely known. This information, however, seems essential for an appropriate management and conservation of these important habitats. In this thesis, we have addressed some of these issues with molecular tools, using DNA barcodes and developing a set of microsatellite markers in corallines. In particular, this thesis pursued a number of objectives:

- To assess the actual diversity of maerl-forming species in European Atlantic beds. We cannot properly assess to what extent the maerl beds found in one area of Atlantic Europe (e.g. French Brittany) are equivalent to those found in other areas (e.g. NW Iberia) without an accurate knowledge of the actual number of maerl-forming species found in each region.
- To investigate the sexual reproductive traits in a major maerl-forming species along the Atlantic European coast: *Phymatolithon calcareum.* The sexual stage in the life cycle of *P. calcareum* has been notably elusive. Moreover, its conspicuously heteromorphic nature may cast some doubts on the actual identity of its collections. New molecular tools (e.g. DNA barcodes) may help to solve these doubts by providing an unambiguous identification.
- To develop species-specific molecular markers with appropriate resolving power for population studies (i.e. microsatellites) in *P. calcareum* using the 454 NGS technology for after applying them to several *P. calcareum* populations from Atlantic European maerl beds. Genetic diversity and population/bed interconnectivity have been largely overlooked in maerl studies, possibly due to a lack of appropriate tools. Again, recent new approaches to microsatellite isolation (NGS technology) have greatly facilitated the acquisition of population genetics markers in non-model organisms that may help to address these issues.

RESULTS

CHAPTER 1

A multilocus species delimitation reveals a striking number of species of coralline algae forming maerl in the OSPAR maritime area

This chapter is based on content published in:

Pardo C., Lopez L., Peña V., Hernández-Kantún J., Le Gall L., Bárbara I. & Barreiro R. (2014). A multilocus species delimitation reveals a striking number of species of coralline algae forming maerl in the OSPAR maritime area. PLoS ONE 9, e104073 (p.12).

A MULTILOCUS SPECIES DELIMITATION REVEALS A STRIKING NUMBER OF SPECIES OF CORALLINE ALGAE FORMING MAERL IN THE OSPAR MARITIME AREA

ABSTRACT

Maerl beds are sensitive biogenic habitats built by an accumulation of loose-lying, non-geniculate coralline algae. While these habitats are considered hot-spots of marine biodiversity, the number and distribution of maerl-forming species is uncertain because homoplasy and plasticity of morphological characters are common. As a result, species discrimination based on morphological features is notoriously challenging, making these coralline algae the ideal candidates for a DNA barcoding study. Here, mitochondrial (COI-5P DNA barcode fragment) and plastidial (*psb*A gene) sequence data were used in a two-step approach to delimit species in 224 collections of maerl sampled from Svalbard (78º96'N) to the Canary Islands (28º64'N) that represented 10 morphospecies from four genera and two families. First, the COI-5P dataset was analysed with two methods based on distinct criteria (ABGD and GMYC) to delineate 16 primary species hypotheses (PSHs) arranged into four major lineages. Second, chloroplast (*psbA*) sequence data served to consolidate these PSHs into 13 secondary species hypotheses (SSHs) that showed biologically plausible ranges. Using several lines of evidence (e.g. morphological characters, known species distributions, sequences from type and topotype material), six SSHs were assigned to available species names that included the geographically widespread *Phymatolithon calcareum*, *Lithothamnion corallioides*, and *L. glaciale*; possible identities of other SSHs are discussed. Concordance between SSHs and morphospecies was minimal, highlighting the convenience of DNA barcoding for an accurate identification of maerl specimens. Our survey indicated that a majority of maerl-forming species have small distribution ranges and revealed a gradual replacement of species with latitude.

KEYWORDS: ABGD, Atlantic Europe, COI-5P, DNA barcoding, GMYC, *Lithothamnion corallioides*, *Lithothamnion glaciale*, *Phymatolithon calcareum*, *psb*A, Rhodophyta.

INTRODUCTION

Maerl or rhodolith beds are accumulations of slow-growing, unattached non-geniculate (non-articulated) coralline algae that build three-dimensional habitats (Adey & McKibbin 1970) that accommodate a wide biodiversity and are, therefore, considered as hotspots of marine life (Peña *et al.* 2014a). Commercial dredging together with a range of indirect impacts (bottom-fishing, aquaculture, eutrophication, sediment dredging) are known to negatively affect their conservation and structure (BIOMAERL Team 2003). As a result, maerl beds are listed as threatened and/or declining habitats by OSPAR (The Convention for the Protection of the marine Environment of the North-East Atlantic; Hall-Spencer *et al.* 2010) and treated as Special Areas of Conservation by European Union (EU) Habitats Directive (Annex I, categories ''sandbank covered by seawater all the time'' and ''large shallow inlets and bays''). In addition, the two coralline algal species commonly regarded as the main constituents of maerl beds in Europe (*Phymatolithon calcareum* (Pallas) W.H. Adey & D.L. McKibbin and *Lithothamnion corallioides* (P.L. Crouan & H.M. Crouan) P.L. Crouan & H.M. Crouan, **Fig. 1**) are listed in Annex V as species whose eventual exploitation must be compatible with maintaining a favourable conservation status.

Maerl beds are widely distributed along the coasts of the North-East Atlantic protected by the OSPAR Convention (OSPAR maritime area) and the adjacent Macaronesia. They are particularly frequent in Scotland, Ireland, Brittany and Galicia (Hall-Spencer *et al.* 2010) at depths ranging from the intertidal to 50 m, but they reach up to 60 m in the Canary Islands and Madeira (Cabioch 1974, Afonso-Carrillo & Gil-Rodríguez 1982, Peña & Bárbara 2009, Hall-

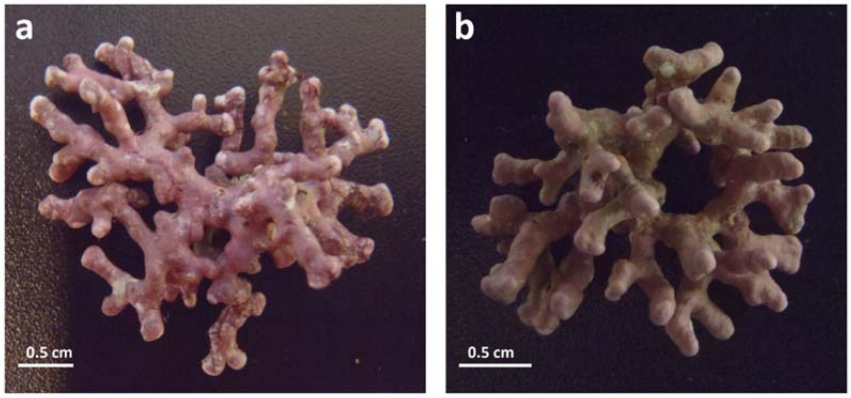


Fig. 1. Maerl-forming species listed in the Annex V of the EU Habitats Directive. **a**. *Phymatolithon calcareum* (voucher CPVP-912, Baie de Morlaix-Brittany, France). **b**. *Lithothamnion corallioides* (voucher CPVP-794, Baie de Douarnenez-Brittany, France).

Spencer *et al.* 2010). Up to 24 species of maerl have been recorded along the OSPAR area and southern adjacent regions (Madeira Archipelago and Canary Islands; **Table 1**). However, the actual number might be smaller as the taxonomic validity of eight taxa seems dubious because they were only reported in pioneer works from the 19th century and early 20th (*Lithothamnion breviaxe* Foslie, *L. fornicatum* Foslie, *L. fruticulosum* (Kützing) Foslie, *L. intermedium* Kjellman, *L. nodulosum* Foslie, *L. norvegicum* (Areschoug) Kjellman, *L. tusterense* Foslie and *L. ungeri* Kjellman). Maerl-forming algae belong to six genera (*Lithothamnion*, *Lithophyllum*, *Mesophyllum*, *Neogoniolithon*, *Phymatholithon* and *Spongites*) from two families (Corallinaceae and Hapalidiaceae) within the orders Corallinales and Hapalidiales (Rhodophyta). According to the literature, four species are widely distributed and seemingly follow a latitudinal replacement cline: *Lithothamnion tophiforme* (Esper) Unger and *L. glaciale* Kjellman are mostly arctic and subarctic species, while *P. calcareum* and *L. corallioides* occur from the North and Celtic Seas to Madeira-Canary Islands (*L. corallioides*) or Azores (*P. calcareum*). The remaining 12 species occupy narrower latitudinal ranges. Eight of them were reported for regions with a long tradition of taxonomic surveys: Scotland, Britain, Ireland, and French Brittany (*Lithothamnion lemoineae* Adey, *L. sonderi* Hauck, *Phymatolithon purpureum* (P.L. Crouan & H.M. Crouan) Woelkerling & L.M. Irvine, *Mesophyllum lichenoides* (J. Ellis) Me. Lemoine, *Lithophyllum dentatum* (Kützing) Foslie, *L. duckerae* Woelkerling, *L. fasciculatum* (Lamarck) Foslie and *L. hibernicum* Foslie). The remaining four include species reported for Macaronesia (*Neogoniolithon brassica-florida* (Harvey) Setchell & L.R. Mason, *Lithophyllum crouanii* Foslie, and *Spongites fruticulosa* Kützing) plus *Mesophyllum sphaericum* V. Peña, Bárbara, W.H. Adey, Riosmena-Rodríguez & H.G. Choi, a maerl alga known from a single location in Galicia. An overwhelming majority of the previous studies have entirely relied on traditional practices of taxonomy based on morphological/anatomical characters even though morphological identification of non-articulated coralline algae is challenging because phenotypic plasticity and convergence have resulted in a lack of well-defined diagnostic characters (Steneck 1986). Only very recently, DNA information has been used to identify and delineate European maerl-forming species (Peña *et al.* 2011) shedding light on our fragmentary knowledge on alpha diversity and genuine distribution of maerl-forming species.

Table 1. Distribution of maerl-forming species reported in the literature for OSPAR regions and southern adjacent areas.

	OSPAR region											Macaronesia		
	I Svalbard Archipelago	Greenland	Iceland	I-II Scandinavia	II-III Scotland	Britain	III Ireland	II-IV Brittany	IV Bay of Biscay	Galicia	Portugal	V Azores	Madeira	Canary Islands
Major maerl-forming species														
Lithothamnion tophiforme (Esper) Unger	Hall-Spencer *et al.* (2010)	Unger (1858) Adey *et al.* (2005) Hall-Spencer *et al.* (2010)	Strömfelt (1886) Adey (1971) Gunnarsson (1977)	Foslie (1895) Adey (1971) Hall-Spencer *et al.* (2010)	-	-	-	-	-	-	-	-	-	-
Lithothamnion glaciale Kjellman	Kjellman (1883) Teichert *et al.* (2012)	Rosenvinge (1894) Foslie (1905) Hall-Spencer *et al.* (2010)	Foslie (1905) Adey (1971) Gunnarsson (1977)	Foslie (1895) Foslie (1905) Adey (1971) Hall-Spencer *et al.* (2010)	Foslie (1905) Adey & Adey (1973) Irvine & Chamberlain (1994) Hall-Spencer *et al.* (2010)	Adey & Adey (1973) Irvine & Chamberlain (1994) Hall-Spencer *et al.* (2010)	Adey & Adey (1973) Irvine & Chamberlain (1994)	-	-	-	-	-	-	-
Phyamtolithon calcareum (Pallas) W.H. Adey & D.L. McKibbin	-	-	-	Suneson (1958) Adey (1971) Hall-Spencer *et al.* (2010)	Hall-Spencer & Moore (2000)	Adey & Adey (1973) Woelkerling & Irvine (1986) Irvine & Chamberlain (1994)	Adey & Adey (1973) Irvine & Chamberlain (1994)	Lemoine (1910) Mendoza & Cabioch (1998) Hall-Spencer *et al.* (2010)	Sauriau *et al.* (2012)	Adey & McKibbin (1970) Peña & Bárbara (2004, 2008b)	Peña *et al.* (2009)	Rosas-Alquicira *et al.* (2009)	-	-
Lithothamnion corallioides (P.L.Crouan & H.M.Crouan) P.L.Crouan & H.M.Crouan	-	-	-	Foslie (1895)	-	Foslie (1895) Irvine & Chamberlain (1994) Hall-Spencer *et al.* (2010)	Adey & Adey (1973) Irvine & Chamberlain (1994) Hall-Spencer *et al.* (2010)	Cabioch (1969) Mendoza & Cabioch (1998) Hall-Spencer *et al.* (2010)	-	Adey & McKibbin (1970) Peña & Bárbara (2004)	-	-	Cabioch (1974)	Afonso-Carrillo & Gil-Rodríguez (1982) Hall-Spencer *et al.* (2010)

Minor maerl-forming species (narrow or uncertain distribution)															
Lithothamnion fruticulosum (Kützing) Foslie	-	Foslie (1895)	Strömfelt (1886)	Foslie (1895)	-	Foslie (1895)	Guiry (1978)	-	-	-	-	-	-	-	
Lithothamnion breviaxe Foslie	-	-	-	Foslie (1895)	-	-	-	-	-	-	-	-	-	-	
Lithothamnion fornicatum Foslie	-	-	-	Foslie (1895)	-	-	-	-	-	-	-	-	-	-	
Lithothamnion intermedium Kjellman	-	-	-	Kjellman (1883) Foslie & Printz (1929)	-	-	-	-	-	-	-	-	-	-	
Lithothamnion nodulosum Foslie	-	-	-	Foslie (1895)	-	-	-	-	-	-	-	-	-	-	
Lithothamnion tusterense Foslie	-	-	-	Foslie & Printz (1929)	-	-	-	-	-	-	-	-	-	-	
Lithothamnion ungeri Kjellman	-	-	-	Kjellman (1883) Foslie (1895)	-	-	-	-	-	-	-	-	-	-	
Lithothamnion norvegicum (Areschoug) Kjellman	-	-	-	Kjellman (1883) Foslie & Printz (1929)	Foslie & Printz (1929)	-	-	-	-	-	-	-	-	-	
Lithothamnion lemoineae Adey	-	-	-	-	Irvine & Chamberlain (1994) Hall-Spencer *et al.* (2010)	-	-	-	-	-	-	-	-	-	

Lithothamnion sonderi Hauck	-	-	-	-	Hall-Spencer *et al.* (2010)	-	-	-	-	-	-	-	-	-	-
Phymatolithon purpureum (P.L. Crouan & H.M. Crouan) Woelkerling & L.M. Irvine	-	-	-	Adey & Adey (1973)	-	-	Adey & Adey (1973) Irvine & Chamberlain (1994)	-	-	-	-	-	-	-	-
Lithophyllum duckerae Woelkerling	-	-	-	-	-	Irvine & Chamberlain (1994)	-	-	-	-	-	-	-	-	-
Litthophyllum hibernicum Foslie	-	-	-	-	-	-	Foslie (1906) Irvine & Chamberlain (1994) Hall-Spencer *et al.* (2010)	-	-	-	-	-	-	-	-
Mesophyllum lichenoides (J. Ellis) Me. Lemoine	-	-	-	-	-	-	Lemoine (1913)	-	-	-	-	-	-	-	-
Lithophyllum fasciculatum (Lamarck) Foslie	-	-	-	-	-	Irvine & Chamberlain (1994) Hall-Spencer *et al.* (2010)	Foslie (1899) Irvine & Chamberlain (1994) Hall-Spencer *et al.* (2010)	Cabioch (1969) Hall-Spencer *et al.* (2010)	-	-	-	-	-	-	-
Lithophyllum dentatum (Kützing) Foslie	-	-	-	-	-	-	Foslie (1900) Irvine & Chamberlain (1994) Hall-Spencer *et al.* (2010)	Hall-Spencer *et al.* (2010)	-	-	-	-	-	-	-

Mesophyllum sphaericum V. Peña, Bárbara, W.H. Adey, Riosmena-Rodríguez & H.G. Choi	-	-	-	-	-	-	-	-	-	Peña *et al.* (2011)	-	-	-	-
Neogoniolithon brassica-florida (Harvey) Setchell & L.R. Mason	-	-	-	-	-	-	-	-	-	-	-	Rosas-Alquicira *et al.* (2009)	-	-
Lithophyllum crouanii Foslie	-	-	-	-	-	-	-	-	-	-	-	Rosas-Alquicira *et al.* (2009)	-	-
Spongites fruticulosa Kützing	-	-	-	-	-	-	-	-	-	-	-	-	Cabioch (1974)	-

Records restricted to reports as maerl; some species have wider reported ranges as encrusting forms. Currently accepted names are used except for *Lithothamnion fruticulosum* where an older name is retained (see further details in Woelkerling 1985).

The onset of the 21st century has witnessed notable technological advances that can facilitate and accelerate the description of biodiversity (Wheeler 2008, Puillandre *et al.* 2012b). In particular, DNA barcoding (http://www.ibol.org/) employs short, standardized DNA fragments as a diagnostic tool for identifying species (Hebert *et al.* 2003). In Rhodophyta, DNA barcodes obtained by sequencing the 5' end of the mitochondrial gene cytochrome oxidase I (COI-5P; Saunders 2005, Robba *et al.* 2006) proved very effective to shortcut the difficulties of morphology based identification, allowing an accurate identification of known species (Walker *et al.* 2009, Clarkston & Saunders 2010, Le Gall & Saunders 2010a,b, Rueness 2010, Saunders & McDonald 2010, Sherwood *et al.* 2010a, Mamoozadeh & Freshwater 2012, Peña *et al.* 2014b) and/or the detection of cryptic ones (Saunders 2008, Le Gall & Saunders 2010b, Saunders & McDonald 2010, Kucera & Saunders 2012, Milstein & Saunders 2012). In comparison, COI-5P sequences have been less frequently used to delineate new species of red algae (Walker *et al.* 2009, Clarkston & Saunders 2010, Saunders & McDonald 2010, Mamoozadeh & Freshwater 2012, Milstein & Saunders 2012). Indeed, when DNA barcoding suggested the existence of new species, it was rarely regarded as a definitive proof; instead, it was used along with other genetic, morphological, geographical or ecological features in what has been referred to as integrative taxonomy (de Salle 2006, Wiemers & Fiedler 2007, Puillandre *et al.* 2012b).

Despite the above, DNA barcodes have been used as an exploratory tool for poorly surveyed taxa provided that the groups delineated by barcodes are regarded as primary species hypothesis (PSHs; Pons *et al.* 2006, Puillandre *et al.* 2012a). PSHs can then be further tested with other sources of molecular, morphological, geographical and/or ecological evidence and even a multistep approach has been proposed to turn PSHs into more conclusive secondary species hypotheses (SSHs; Puillandre *et al.* 2012b) (for a similar approach see the molecular-assisted alpha taxonomy in Saunders 2008). In this context, the initial step is crucial and consists of the partition of COI-5P sequences into a set of PSHs. Recently, two methods based on distinct criteria have been proposed to infer the limits of the various PSHs when only molecular data are available and with no need for prior assumptions. On the one hand, the Automatic Barcode Gap Discovery (ABGD; Puillandre *et al.* 2012a) is a fast method that uses distances to split the sequence alignment into a set of PSHs following a recursive procedure until there is no further partitioning. This procedure automatically finds breaks in the distribution of genetic pairwise distances, referred to as the "barcode gap", even when intra- and interspecific distances overlap. On the other hand, the General Mixed Yule Coalescent (GMYC) model (Pons *et al.* 2006) is based on detecting the shift of the branching rate that

takes place in clock-constrained calibrated trees at the point of transition from species-level (speciation) to population-level (coalescence) evolutionary processes. Using a likelihood criterion, the GMYC method permits an automated species delineation with appropriate statistical measures of confidence. A later extension of the method allows for a variable transition from coalescent to speciation among lineages (Monaghan *et al.* 2009). GMYC has been shown to be robust to a range of departures from its assumptions (varying population sizes among species, alternative scenarios for speciation/extinction, population growth and subdivision within species) but the accuracy of its delimitations can be compromised in groups with large effective population sizes and short divergence times between species (Fujisawa & Barraclough 2013). Other potential shortcomings of the GMYC method have been extensively discussed elsewhere (Hamilton *et al.* 2014).

In this study, COI-5P sequences were obtained for maerl-forming species along the OSPAR maritime area and the adjacent Macaronesia. DNA barcodes were used to delimit a set of PSHs that were subsequently corroborated or challenged with independent molecular, geographic, and morpho-anatomical evidence.

MATERIAL AND METHODS

Study area and sample collection

As the study did not involve endangered or protected species, no specific permissions were required for sampling at most locations (see **Table S1** for coordinates). Still, sampling at two locations situated within a national park in NW Spain (lat 42.211º long -8.896º and lat 42.394º long -8.815º) was conducted with the permission of the park authority (Parque Nacional Marítimo-Terrestre das Illas Atlánticas de Galicia) and the park authority has signed a document stating its interest in the results of this study.

Collection information for all the specimens used in this study is available at the Barcode of Life Data Systems (BOLD: www. boldsystems.org; project ‘‘maerl-NE Atlantic’, code MAERL). From 1999 to 2011, maerl specimens were extensively sampled by SCUBA diving or dredging within 4 out of the 5 regions of the OSPAR maritime area (**Table S1**); sampling ranged from the low intertidal to 40 m depth. Despite our efforts, no sample could be obtained for region V where maerl beds are probably restricted to the Azores Archipelago. To circumvent this shortage, samples were collected from the other two Macaronesian Archipelagos: the Canaries and Madeira. Sampling sites included type/neotype localities for 3 out of the 4 widely

distributed maerl-forming species: *L. corallioides* (Rade de Brest, Finistère, France, Crouan & Crouan 1867), *P. calcareum* (Falmouth Harbour, Cornwall, England, Woelkerling & Irvine 1986), and *L. glaciale* (Spitsbergen Island, Svalbard Archipelago, Teichert *et al.* 2012). Additionally, our samples included holotype material of the recently described *M. sphaericum* from the herbarium SANT of Universidade de Santiago de Compostela (Peña *et al.* 2011) and neotype material of *P. calcareum* from the herbarium BM of the British Museum of Natural History (Woelkerling & Irvine 1986, **Fig. 2**).

Freshly collected material was transported to the laboratory in seawater, oven-dried or air-dried as soon as possible, and vouchered in silica. Vouchers were temporarily deposited in a personal collection (BioCost Research Group, Universidade da Coruña, Spain) and will be transferred to the official SANT Herbarium. When feasible, several specimens per morphotype (differences in size, shape, branch thickness, and general habit) were sequenced at each locality. This sampling regime was intended to maximize the detection of species encountered at the various collecting sites while keeping the sequencing effort to a reasonable size. All specimens were photographed and identified to the lowest taxonomic level possible using morphology-based keys and specialized literature. A selection of specimens was also examined under Scanning Electron Microscope (SEM, model JEOL JSM 6400, Universidade da Coruña).

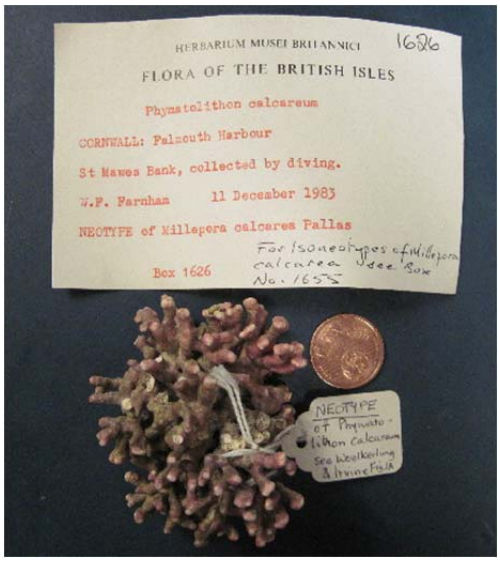


Fig. 2. Neotype of *Phymatolithon calcareum*. Box 1626 (BM000712373), herbarium BM of the British Museum of Natural History (London). Photo: Jazmín J. Hernández-Kantún.

Field identification

A putative species name was assigned to all specimens based on their gross morphology. Our 224 collections were partitioned into 10 different morphospecies belonging to 4 genera and 2 families (Hapalidiaceae and Corallinaceae). Most plants were identified either as *Phymatolithon calcareum* (140 collections) or as *Lithothamnion corallioides* (60), two main constituents of maerl in Atlantic Europe. A much smaller number of collections fitted the description of *Lithothamnion glaciale* (9), *Mesophyllum sphaericum* (4), *Lithophyllum dentatum* (2), and *Lithophyllum fasciculatum* (1). Finally, eight plants exhibited external features typical of the genus *Lithothamnion*; however, none of these plants exhibited diagnostic characters necessary for their identification at the species level. Nevertheless, based on some morphological distinctions, they were partitioned into four morphospecies temporarily labeled as *Lithothamnion* sp.1 (3 collections), *Lithothamnion* sp.2 (1), *Lithothamnion* sp.3 (1), and *Lithothamnion* sp.4 (3).

DNA extraction, PCR amplification and sequencing

A subsample for DNA extraction was obtained by grinding a portion of the living surface of each specimen after avoiding areas with epiphytes, animal structures, and/or damaged tissue. Special cautions were taken with the neotype of *Phymatolithon calcareum* because this specimen has been archived in BM since 1983 (Woelkerling & Irvine 1986). To avoid contamination, this archival specimen was processed (DNA extraction and PCR amplification) individually with fresh batches of reactants on a separate date after carefully cleaning the laboratory. To increase the possibility of detecting contamination, several genes were amplified for this specimen on the same date (SSU, *rbcL*, *psbA*, COI-5P) running negative controls in parallel for each gene; none of the chromatograms showed evidence of background signal and all negative controls were clean (Hughey *et al.* 2001). The holotype of *Mesophyllum sphaericum* in SANT was collected shortly before the present study (October 2008) and processed alongside topotype material of the same species. Attempts to acquire sequence data from type material of other species included *Lithothamnion corallioides*, *Lithothamnion fornicatum*, *Neogoniolithon brassica-florida*, and *Spongites fruticulosa* but proved unsuccessful.

DNA was extracted with the DNeasy Blood & Tissue Kit Spin-Column Protocol (Qiagen) following manufacture's recommendations. Two gene fragments were amplified: (i) a fragment of 664 bp of the standard DNA barcode (the 5' end of the mitochondrial gene

cytochrome oxidase I, COI-5P) with primers GazF1 and GazR1 from Saunders (2005), and GCorR3 (5'TGATTYTTYGGACATCCTGA3'), and (ii) a fragment of 892 bp of the plastidial gene photosystem II reaction center protein D1 (*psb*A) with primers *psb*A-F1 and *psb*A-R2 from Yoon *et al.* (2002). PCR reactants were prepared in a laminar flow hood and PCRs were performed in 25 mL 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 Taq DNA Polymerase (Sigma-Aldrich) in a Biometra TProfesional Basic thermocycler following Saunders & McDevit (2012b). Amplification success was evaluated by electrophoresis. After removing the excess of primers and nucleotides with shrimp alkaline phosphatase and exonuclease I enzymes, PCR products were bidirectionally sequenced at Macrogen facilities (http://www.macrogen.com). All sequences are publically available in BOLD and GenBank databases (see **Table S1** for BOLD Process IDs and GenBank accession numbers).

Data analyses

Sequences were aligned and edited using the program Geneious v.5.6.6. As we aimed to delimit species based on sequence data rather than to assess their phylogenetic relationships, we chose not to run maximum-likelihood or maximum parsimony analyses. Instead, COI-5P sequences were partitioned into a set of PSHs using two bioinformatics tools: ABGD (Puillandre *et al.* 2012a) and GMYC (Pons *et al.* 2006, Monaghan *et al.* 2009). For ABGC, genetic distances between specimens were calculated using the Kimura two Parameters (K2P) model, a standard metric in DNA barcoding studies. ABGD was remotely run at http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html using default values except for the relative gap width (*X*) which was set to 10 to avoid the capture of small local gaps. For the GMYC method, duplicate haplotypes were removed from the alignment using DnaSP v.5.10.01 (Librado & Rozas 2009). Since the GMYC method is based on branching rates, branch lengths were estimated under a relaxed log-normal clock with the Bayesian analysis implemented in BEAST v.1.7.4. Following Monaghan *et al.* (2009), BEAST was run using a coalescent (constant population size) prior and the best-fitting model identified by jModelTest (HKY+G with G=0.153) (Guindon & Gascuel 2003, Darriba *et al.* 2012); the parameters for the substitution model (substitution rate, rate heterogeneity, and base frequencies) were unlinked across positions. MCMC chains were run for 20 million generations with a 10% burnin (determined by visual inspection of MCMC progression). After termination, the MCMC output was analyzed with TreeAnnotator v.1.7.4 using all trees after the burnin, a posterior probability limit of 0.5, targeting the maximum clade credibility tree, and keeping the target node heights. Both the

single-threshold and the multiple-threshold versions of the GMYC model (Pons *et al.* 2006, Monaghan *et al.* 2009) were optimized onto the output tree with the help of the SPLITS v.1.0-19 package for R. AIC-based support values for the GMYC clusters were calculated following Fujisawa & Barraclough (2013). BEAST and TreeAnnotator were also employed to reconstruct a phylogeny for the *psb*A gene with the same options used for the COI-5P sequences but a different best-fitting model (GTR+G with G= 0.175).

RESULTS

Primary Species Hypothesis delineation based on COI-5P sequence data

The 224 collections of maerl were sequenced for a 664 bp fragment of the barcoding COI-5P gene; 29 unique haplotypes were found with 227 variable sites. Genetic pairwise K2P distances ranged from 0 to 0.21 while the shape of the pairwise distance distribution was clearly bimodal with two conspicuous peaks at pairwise distances < 0.01 and 0.14–0.16 separated by a rough gap of very low frequencies (**Fig. S1**). The number of PSHs delineated with the ABGD method varied with the maximum prior distance (*P*) used in the analysis (**Fig. S2**). Extreme prior thresholds led to uninformative partitions where either each haplotype was delimited as a different species or all haplotypes were included into a single PSH. Intermediate values of *P* led to partitions with 9 (*P* = 0.013), 13 (*P* = 0.008) and 14 PSHs (*P* = 0.0017 to 0.005). Partitions with 9 and 14 PSHs are detailed in **Fig. 3**.

GMYC was applied to a phylogenetic tree reconstructed with a relaxed lognormal clock. Effective sample size for each statistic of the tree always was > 500 and the MCMC converged to a stationary distribution. The likelihood of the null model (L_0= 170.19) was significantly lower than the maximum likelihood of the single-threshold version of GMYC model (L_{single}= 177.97, *P*-value = 0.0004). According to the latter, the transition from speciation to coalescent occurred at a depth of 0.0014 substitutions per site and resulted in a partition with 13 PSHs (confidence interval 4–14): 7 distinct clusters plus 6 singletons (**Fig. 3**). The likelihood of the multiple-threshold version of the model ($L_{multiple}$= 178.36) also was significantly higher than that of the null model (*P*-value = 0.00028). This version detected a second threshold for the speciation-coalescent transition towards the tips of the tree at an extremely shallow depth of only 0.00026 substitutions per site. With this new threshold, the analysis delimited 15 PSHs (confidence interval 4–15): 7 clusters plus 8 singletons. The mean support value across GMYC clusters was similar in the single-threshold (0.73 ± 0.138) and in the multiple-threshold (0.74 ±

0.237) methods. Nonetheless, the two new clusters delimited by the multiple-threshold algorithm had very little support (< 0.45).

Although based on entirely different criteria, the partitions delineated by ABGD and GYMC were notably congruent. The less inclusive partition obtained by ABGD (14 PSHs) was nearly identical to the one produced by the single-threshold version of the GMYC model (13 PSHs). The only discrepancy involved haplotype Hap_24 (a specimen from Svalbard Archipelago) which was resolved as a singleton by ABGD while the GMYC model clustered it with other collections from Svalbard and Scandinavia (Hap_7). In comparison, the more inclusive partition of ABGD (9 PSHs) and the multiple-threshold GMYC (15 PSHs) showed more discrepancies. However, their conflicting PSHs (greyed in **Fig. 3**) seemed biologically implausible because (i) the more inclusive hypothesis of ABGD clustered groups of haplotypes separated by average K2P distances as large as 0.064 (PSHs 4 to 7 in **Fig. 3**) or 0.081 (PSHs 10 to 12), and (ii) the conflicting PSHs in the multiple-threshold version of GMYC were separated by distances as small as 0.002–0.005 (sequence divergence between and within PSHs is shown in **Table S2a**).

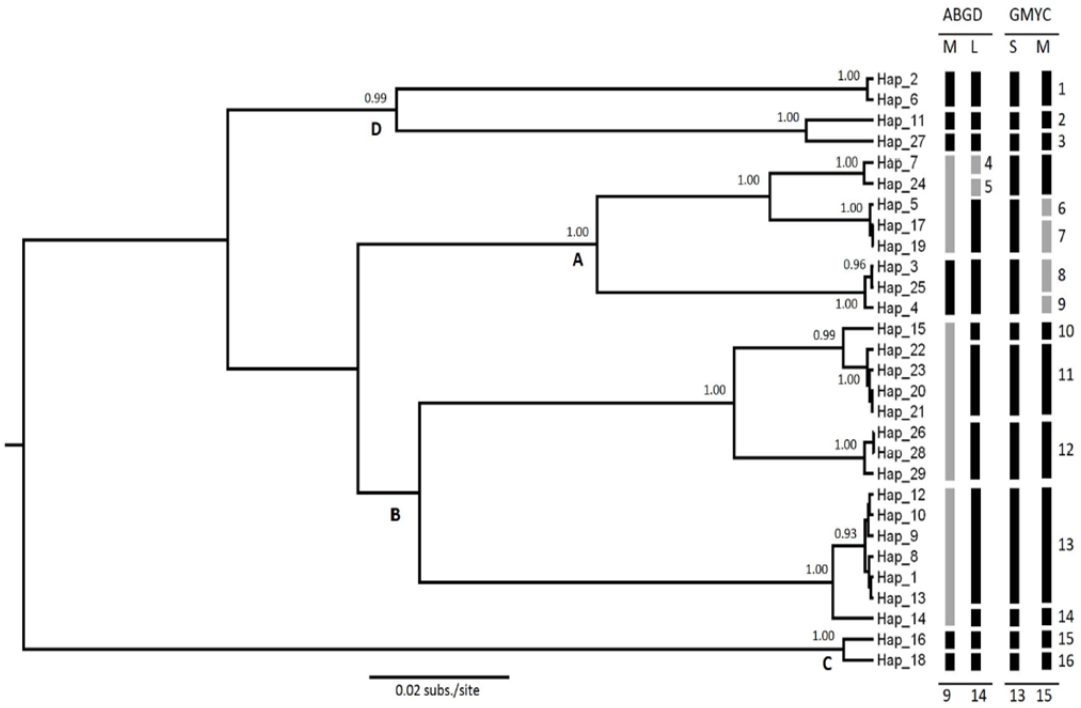


Fig. 3. Primary Species Hypothesis (PSHs) delineated with the COI-5P gene. Bayesian gene tree with posterior probabilities (> 0.9) next to each node. Branch tips are the 29 haplotypes detected in the study. Vertical thick lines indicate PSHs delineated with ABGD and GMYC methods; numbers next to the vertical lines are PSH codes; letter A-D next to some nodes indicate major lineage. For ABGD, partitions for the more inclusive (M) and less inclusive (L) results are shown. GMYC partitions include the single-threshold (S) and multiple-threshold (M) variants of the method. Grey thick lines indicate discrepancies between partitions.

COI-5P and psbA phylogenies and Secondary Species Hypothesis

The phylogeny inferred from the mitochondrial COI-5P gene was well resolved (15 nodes with posterior > 0.95 out of 26 nodes) and four major lineages could be distinguished (**Fig. 3**). Regardless of the approach used for species delineation, all the PSHs that included more than one COI-5P haplotype always coincided with clades with high statistical support (posterior > 0.9) with the exception of PSH 7.

The chloroplastic *psbA* gene was sequenced for fifteen of the sixteen PSHs defined with COI-5P data. A fragment of 892 bp generated for 29 specimens produced 15 haplotypes. The phylogeny inferred from *psb*A data was remarkably congruent with the one inferred from the COI-5P gene (**Fig. 4**). Again, four major lineages could be recognized, one of them separated from the others at an earlier time. Eleven PSHs were characterized by unique *psb*A haplotypes. The remaining four PSHs shared *psb*A haplotypes by pairs (PSH 4–5, PSH 6–7); these pairs corresponded to those cases where the delineation produced by ABGD was in conflict with the solution of the GMYC method. Most *psb*A haplotypes exhibited pairwise distances within a range of 10 (equivalent to 98.8% similarity) to 113 differences (86.6%). Still, a few PSHs were characterized by *psb*A sequences separated by distances as small as 2 (PSH 13 *vs.* PSH 14, 99.8% similarity), 3 (PSH 15 *vs*. PSH 16, 99.6%) or 5 point mutations (PSH 10 *vs.* PSH 11, 99.4%). Only two PSHs (PSH 3 and PSH 4) produced more than one (two) *psb*A haplotypes that were separated by a single mutation (99.9% similarity).

Given the consistency between the two phylogenies, any PSH drawn using ABGD and GMYC that was either monophyletic for *psb*A or had unique *psb*A haplotypes was proposed as a SSH. As a result, 16 initial PSHs were converted to 13 SSHs (**Fig. 4**; see also **Table S2b** for average COI-5P sequence divergence between and within SSHs). Two pairs of PSHs alternatively recognized as either a single PSH or as two different PSHs by ABGD and GMYC were turned in a single SSH each (SSH 4+5, SSH 6+7). Unfortunately, we did not manage to obtain a *psb*A sequence for PSH 9, a PSH supported by only one of the partitions derived from the GMYC model. Since its COI-5P sequence was very close to the haplotypes found in PSH 8, we opted for a conservative inclusive approach and considered these two PSHs as a single SSH (8+9). The final partition into SSHs matched the delineation obtained with the single-threshold alternative of GMYC applied to COI-5P data only and was nearly identical to the less inclusive partition generated by ABGD.

Matches in public data bases

Based on the literature and on the magnitude of intra-SSH variability found in our study, we used ad-hoc cutoff values (> 98% identity for COI-5P, > 99% for *psbA*) to determine which GenBank searches had returned hits for potential conspecifics. Only 9 out of our 29 COI-5P haplotypes (4 SSHs) and 3 out of the 15 *psb*A haplotypes (2 SSHs) resulted in a relevant match in either GenBank or BOLD (**Table S3**). Altogether, we obtained hits for 4 out of our 13 SSHs: SSH4+5, SSH6+7, SSH12, and SSH16. Only SSH4+5 resulted in a match to an identified species, *L. glaciale*: our COI-5P sequences were 98.6–99.9% similar to, and shared the same Barcode Index Number (BIN BOLD:AAA6958), 39 accessions uploaded to BOLD for plants collected in Northeast USA and Canada. Also from OSPAR region I, SSH6+7 was conspecific with plants (BIN BOLD: ABA9580) from the Pacific (British Columbia) which, according to pictures logged in BOLD, have a branched morphology typical of maerl-forming plants. Finally, SSH12 and SSH16 had conspecific matches in GenBank with specimens from Brittany, France, which were only identified as Corallinales.

Concordance with morphological idenfitication

The total number of species identified based on their morphological features (10) was close to the number of SSHs (13) delimited with molecular data. However, among the 11 SSH with more than one specimen, only SSHs 1, 3, 11, and 13 were consistently assigned to a single morphospecies (**Fig. 4**). Many morphospecies contained collections from two, five or even six distinct SSHs. The only exceptions were *Mesophyllum sphaericum* (**Fig. 4**), a maerl species with a distinctive spherical morphology, and the three collections assigned to morphospecies *Lithothamnion* sp.2 (**Fig. 4**) that clustered under SSH2.

Attribution of available species names

Six SSHs could be assigned to a species name using a body of proofs. In two cases, name assignment rested on comparisons with molecular data obtained from type material. SSH 12 was identical to COI-5P sequences obtained from neotype material of *Phymatolithon calcareum* from BM. Likewise, our collections of SSH1 included the holotype of *Mesophyllum sphaericum*.

For the second most widespread and common species in our study (SSH8+9), we tentatively attributed the species name *Lithothamnion corallioides* because the latter, together with *Phymatolithon calcareum*, is typically regarded as a common component of maerl beds in

Atlantic Europe. Furthermore, samples from the type locality of *L. corallioides* (Rade de Brest, Finistère, France) (Crouan & Crouan 1867) were resolved in the SSH8+9.

In light of the morphological traits observed by SEM together with the existence of previous records from the same area, we temporarily attributed the names *Lithophyllum fasciculatum* and *L. dentatum* to SSH15 and SSH16. In doing so, we used two names currently available in the literature for the European Atlantic while acknowledging that they belong to entities that need revision. A reassessment of the lectotype of *L. fasciculatum* has revealed that the epithet *fasciculatum* was misapplied to Atlantic plants belonging to the genus *Lithophyllum* (Woelkerling & Lamy 1998). Likewise, it seems unlikely that the coralline algae identified as *L. dentatum* in the Atlantic and their Mediterranean counterparts may be conspecifics (Irvine & Chamberlain 1994). Indeed, the Atlantic plants of *L. dentatum* were previously considered a form of *L. incrustans* Philippi (Lemoine 1913).

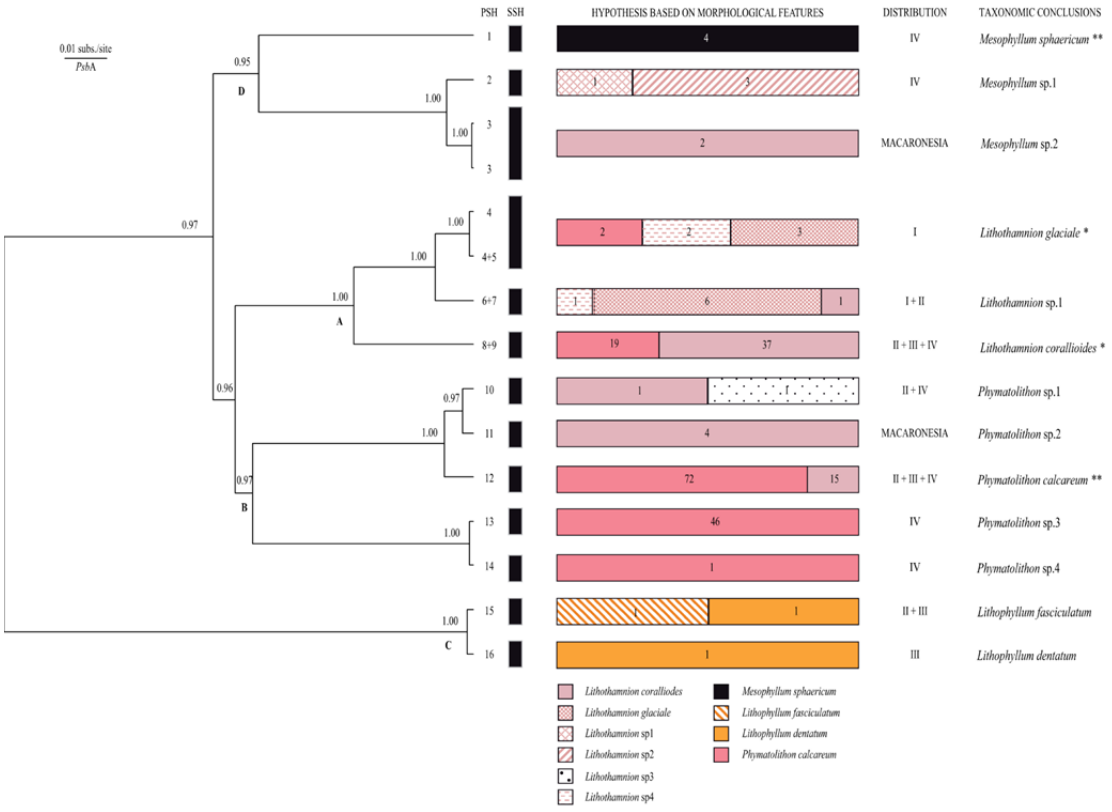


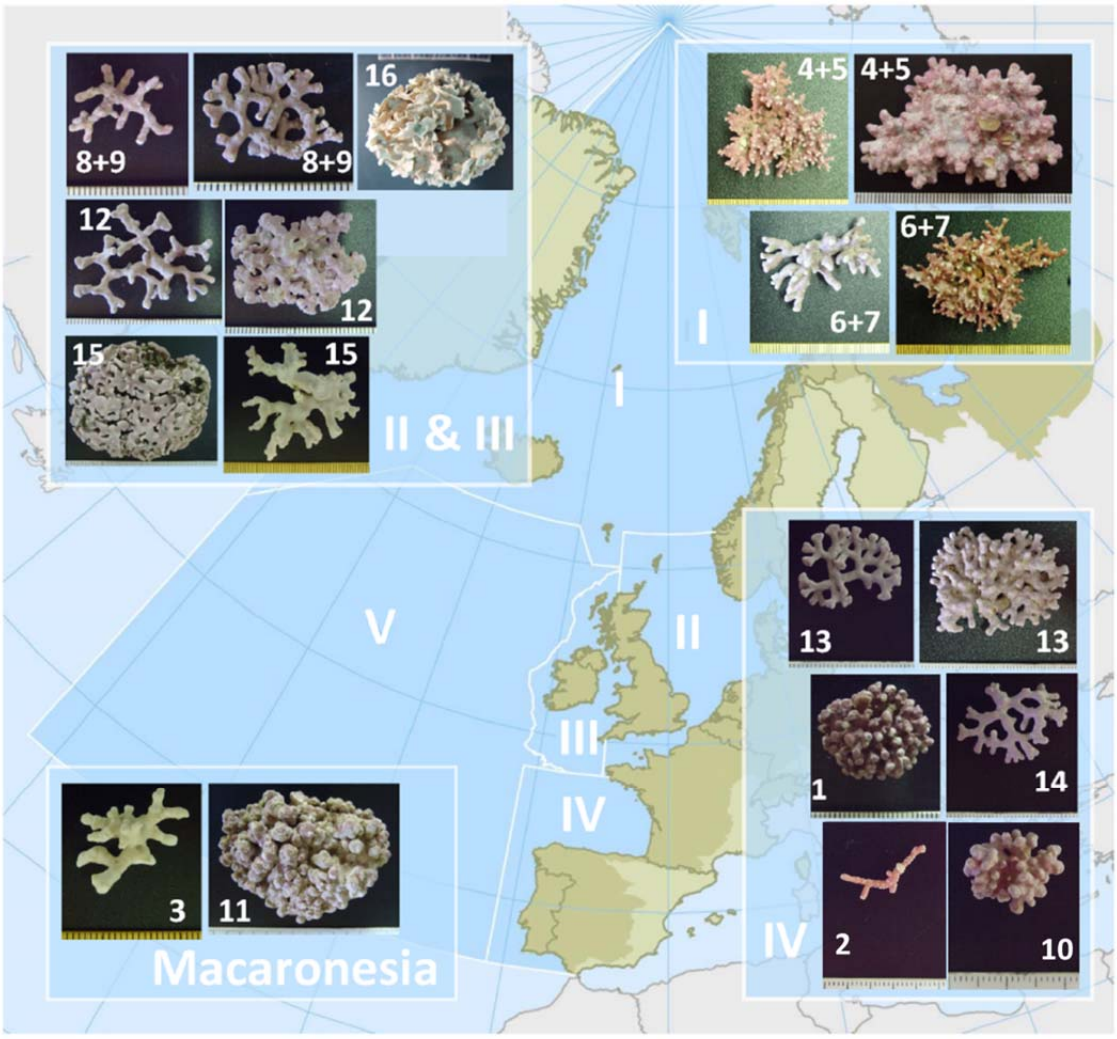
Fig. 4. Secondary Species Hypothesis (SSHs) corroborated with the *psb*A gene. Baseyian gene tree posterior probabilities (> 0.9) next to each node. Branch tips are the 16 *psb*A haplotypes detected in the study. Numbers at the tip of the branches are Primary Species Hypothesis (PSH) code (see **Fig. 3**) while vertical thick lines delineate SSHs; letters A-D next to some nodes indicate major lineages. Stacked horizontal bars nexrt to the gene tree indicate the morphospecies identified at the onset of the study; numbers within bars are the actual number of specimens recorded for each morphospecies. The distribution of each SSH across OSPAR regions and its taxonomic identity is also provided. * topotype specimens sequenced, ** type specimen sequenced.

Finally, we attributed the species name *Lithothamnion glaciale* to SSH4+5 based on the result of the BOLD identification engine. The name used in BOLD has not been confirmed by matching to sequences of type material (see Hind & Saunders 2013a) and should be used with caution. Nonetheless, we also recorded SSH4+5 in Spitsbergen Island (Svalbard Archipelago), the type locality of *L. glaciale* and where this coralline is reported to be common along the west and north coasts of the island (see Teichert *et al.* 2012 and references therein). We did not dare to link other SSHs to available species names and, therefore, seven SSHs were left without a binomial name. Nevertheless, their generic affiliations were evident based on their morphological traits and the phylogenetic relationships inferred from our COI-5P and *psb*A data, and we temporarily named them as *Lithothamnion* sp.2 (SSH6+7), *Phymatolithon* sp.1 (SSH10), *Phymatolithon* sp.2 (SSH11), *Phymatolithon* sp.3 (SSH13), *Phymatolithon* sp.4 (SSH14), *Mesophyllum* sp.1 (SSH2), and *Mesophyllum* sp.2 (SSH3).

Geographical distribution

A majority of SSHs were restricted to one (six SSHs) or two (four SSHs) sampling areas (**Fig. 5**). The remaining three SSHs showed wider, largely continuous distributions. *Lithothamnion* sp.2 (SSH6+7; for equivalence between SSHs and species names see **Fig. 4**) seemed confined to high latitudes in OSPAR regions I and II where it showed minimal overlap with other maerl-forming plants except the phylogenetically close *L. glaciale* (SSH4+5). Southward, *Lithothamnion* sp.2 was replaced by two species, *L. corallioides* (SSH8+9) and *P. calcareum* (SSH12), with wide ranges that reached northwest Spain. None of the species found in Macaronesia (Madeira and Canary Islands) was detected in the OSPAR area and the other way around.

Each major lineage resolved in our phylogeny had different ranges and limits. *Lithothamnion* (lineage A, **Fig. 5**) was detected at mid to high latitudes in the OSPAR area, with a southern edge in northwest Spain. *Phymatolithon* (Lineage B) went unrecorded in OSPAR region I; instead, it reached the warmer coasts of southern Portugal and Macaronesia, albeit with a replacement of species. Finally, *Lithophyllum* (lineage C) was restricted to the British Isles and north of France whereas *Mesophyllum* (lineage D) was confined to southern latitudes (Spain, Portugal and Macaronesia).



Region	Country	4+5	6+7	8+9	12	13	14	10	11	15	16	1	2	3
OSPAR I	Svalbard	3												
	Norway	4	1											
	Iceland		3											
OSPAR II	Norway		1		1									
	UK (South)				2									
	France (North)			8	13			1		1				
OSPAR III	Ireland/UK (West)		3	23	21					1	1			
OSPAR IV	France (West)			11	6									
	Spain			14	44	37						4		
	Portugal					9	1	1					4	
Macaronesia									4					2
	Lineage	A: Lithoth			B: Phymat					C: Lithoph		D: Mesoph		

Fig. 5. Morphological variability and distribution of the Secondary Species Hypotheses (SSHs) along the OSPAR area. The table indicates the number of specimens assigned to each SSH per OSPAR region and country. Vertical lines delimitate four major lineages revealed by both COI-5P and *psb*A phylogenies; tentative names are provided for the clades (genus level) based on sequence information from type/topotype material and on the occurrence of conspicuous morphological features. Lithoth = *Lithothamnion*, Phymat = *Phymatolithon*, Lithoph = *Lithophyllum*, Mesoph = *Mesophyllum*. Scale divisions in the photos are mm.

DISCUSSION

Delimitation and identification of species of coralline algae forming maerl

Our spatially comprehensive sampling likely provides a thorough picture of the alpha diversity of maerl along European Atlantic coasts. Our analyses of both COI-5P and *psb*A sequence data have delineated 13 SSHs, a number comparable to the 16 species reported for the OSPAR regions and Macaronesia (see **Table 1** for references). Nevertheless, linking available binomial names to the SSHs uncovered in our study was a delicate task. In addition to various analyses of the sequence data, additional evidence (morpho-anatomical observations, previously known species distribution, molecular data from type and topotype specimens) was required to guide our decisions at the time of attributing names. Following this approach, we managed to name almost half of the species detected in our study with acceptable confidence. However, it is likely that most, if not all, of the species that we left unnamed in this study may have already been described elsewhere.

We did not dare to identify SSH6+7 (*Lithothamnion* sp. 1) to species level. Its confinement to OSPAR region I and to northernmost sites of region II suggests that *L. tophiforme* could be a plausible name but our sequences did not match two collections from New Foundland, Canada lodged in BOLD as *L. tophiforme*. However, the identity of these collections in BOLD has not been confirmed with sequence data from type material and should be considered with caution. *Lithothamion tophiforme* is mainly reported as an Arctic species that, in European waters, is confined to very high latitudes (Adey *et al.* 2005). Somewhat unexpectedly, however, *L. tophiforme* went unrecorded in a detailed recent investigation of the northernmost maerl communities currently known, discovered in 2006 at 80º31'N in the Svalbard Archipelago (Teichert *et al.* 2012). Instead, these communities were dominated by *L. glaciale*, the only species that we found in our collections from Svalbard. Interestingly, our BOLD searches revealed that *Lithothamnion* sp. 1 also occurs in the North Pacific (British Columbia). A comparable circumpolar distribution has been reported for *L. glaciale* (see references in Guiry & Guiry 2015). Our results show that *Phymatolithon* sp.3 (SSH13) is a major, even dominant, component of maerl beds in Spain and Portugal. Indeed, a recent quantitative study with DNA barcodes demonstrates that the widespread belief that *L. corallioides* and *P. calcareum* are the major builders of maerl in the temperate European Atlantic does not hold for the Iberian Peninsula. Instead, they are gradually replaced by *Phymatolithon* sp.3 in Galicia (N-W Spain) to become extremely rare in S Portugal (Carro *et al.*

2014). Despite our efforts, we have been unable to resolve the identity of this species beyond generic level. The examination by SEM revealed traits also found in *Phymatolithon lamii* (e.g. sunken, rimless conceptacles), a common coralline throughout the British Isles, northern Spain, France, Norway, Iceland and eastern North America (Chamberlain 1991). It has also been reported from the western North Pacific Ocean (Zhakova 1985, Perestenko 1994) and, more recently, from the Mediterranean where it might be an alien species (Kaleb *et al.* 2012). In addition, one of the co-authors (V.P., unpublished results) recently sequenced a 600 bp long fragment of the *psb*A gene from the type specimen of *Phymatolithon lamii* (Me. Lemoine) Y.M. Chamberlain (herbarium PC from Muséum National d'Histoire Naturelle, Paris, France) that reveals a low-moderate divergence with our *Phymatolithon* sp.3. However, *P. lamii* has always been described as encrusting thalli, and there is no previous record of its occurrence as maerl. Hence, further sequence data from type material will be required to assign a species name to SSH13. Lastly, our results clearly indicate that the maerl-forming algae that colonize Macaronesia deserve further study with appropriate sampling design and molecular tools.

To our knowledge, this is the first barcoding study focused on maerl-forming algae. Previous DNA barcode studies on coralline algae mostly focused on geniculate forms (Robba *et al.* 2006, Walker *et al.* 2009, Hind & Saunders 2013a,b) or were intended to resolve infra-ordinal phylogenetic relationships among the Corallinales (Bittner *et al.* 2011). Nevertheless, Bittner *et al.* (2010) sequenced geniculated and non-geniculated coralline algae, mainly from South Pacific Islands, for *psb*A and COI-5P, and used ABGD and GYMC to delineate species. As the authors found very divergent numbers of "genetic species" depending on the criteria and the marker, they concluded that DNA-barcoding was non-accurate for assessing the species diversity in this group (but see Hamilton *et al.* 2014 for alternative explanations when GMYC has a highly divergent outcome). Contrarily, we are in favor of an integrative systematic approach to investigate the diversity of maerl-forming red algae. We propose the use of the mitochondrial COI-5P barcode as the first marker and the plastidial *psb*A gene as a secondary marker along with other lines of evidence. In this regard, we follow other authors that already noted the intrinsic limitations of delimiting species from single-locus studies and advocate the incorporation of multiple lines of evidence (biogeographical, biological, additional gene sequences) in this studies (Puillandre *et al.* 2012b, Fujisawa & Barraclough 2013, Hamilton *et al.* 2014).

Despite considerable efforts to identify specimens collected in this study based on their morpho-anatomical characters, sequence data were incomparably more efficient. In fact, our

Fig. 4 shows that even maerl assigned to different morphogenera turned out to belong to the same molecular entity. Initially, this considerable discrepancy between the morphological identification and the molecular-based delimitation might seem astonishing. However, it is just another example of the considerable challenge of discriminating species in a group that largely lacks diagnostic features but shows considerable morphological plasticity and convergence (**Fig. S3a** and **Fig. S3b**). Species delimitation with molecular markers is known to perform well with deeply divergent taxa such as those found in our study. However, we still uncovered a few closely related species that deserve further studies. It is increasingly acknowledged that a multi-locus approach can define reliable species boundaries in those cases (Pons *et al.* 2006, Monaghan *et al.* 2009, Dupuis *et al.* 2012, Puillandre *et al.* 2012b, Payo *et al.* 2013). Even for the more closely related SSHs, the genealogical concordance observed between loci located in different genomes lends additional support to our partition. Finally, the congruence between partitions recovered with analyses based on radically different criteria (coalescence *vs.* distribution of pairwise differences between sequences) indicates that there is a strong signal in the COI-5P data set. If any, the only shortcoming encountered in the course of that study was the erratic amplification of the COI-5P fragment that failed on 1/3 of the specimens at the first attempt, although repeated amplifications and/or re-extracting DNA from the same individual often solved this issue. The remarkable coincidence between the patterns revealed by our *psb*A and COI-5P sequence data indicates that the plastidial marker, which is easier to amplify than COI-5P, could be a useful alternative for the identification of maerl-forming species (but see Hind & Saunders 2013b for a intrageneric study where the gene trees produced by COI-5P and by another plastid-encoded gene, rbcL, were not always congruent). Compared to the standard DNA barcode, *psb*A has lower resolution. However, most of the maerl-forming taxa studied in the OSPAR region are deeply divergent. The only exceptions were two pairs of closely related species with low levels of interspecific divergence (2–3 nucleotide substitutions) that could be mistaken for intraspecific variability (*Lithophyllum fasciculatum vs. L. dentatum*; *Phymatolithon* sp.3 *vs. Phymatolithon* sp.4).

Species distribution and implication for future prospects

Our study reveals that two species of coralline algae are the main constituent of maerl beds in temperate European Atlantic: *Lithothamnion corallioides* and *Phymatolithon calcareum*. Another *Phymatolithon* (i.e. *Phymatolithon* sp.3-SSH13) replaces them in the south while the cold OSPAR region I seems dominated by two species of *Lithothamnion* (*L. glaciale* and *Lithothamnion* sp.1-SSH6+7). The remaining species unraveled in our study are either

infrequent and/or confined in space. The gradual replacement of species with latitude (**Fig. 5**) is consistent with the patterns of maerl distribution reported in the literature (see **Table 1** and references therein). The distribution of coralline algae forming maerl in our study is likewise consistent with a general pattern observed in many taxonomic groups where a majority of species have small geographic ranges whereas a few have large ones (Gaston 1996). Biogeographical distribution patterns of species are strongly controlled by climate (Pearson & Dawson 2003, Bartsch *et al.* 2012). For instance, the confinement of *L. glaciale* to arctic and subarctic locations has been attributed to the fact that this plant only produces reproductive conceptacles when water temperatures are below 9ºC in winter (Wilson *et al.* 2004). Maerl forming coralline algae are likely to be affected by the ongoing global warming (IPCC 2007). They may migrate to regions where the climatic conditions are suitable for their physiology or may become extinct. In that context, our study provides an assessment of genuine distribution of maerl species as well as an efficient tool to monitor putative shifts in southern and northern ranges of each species delineated.

SUPPLEMENTAL DATA

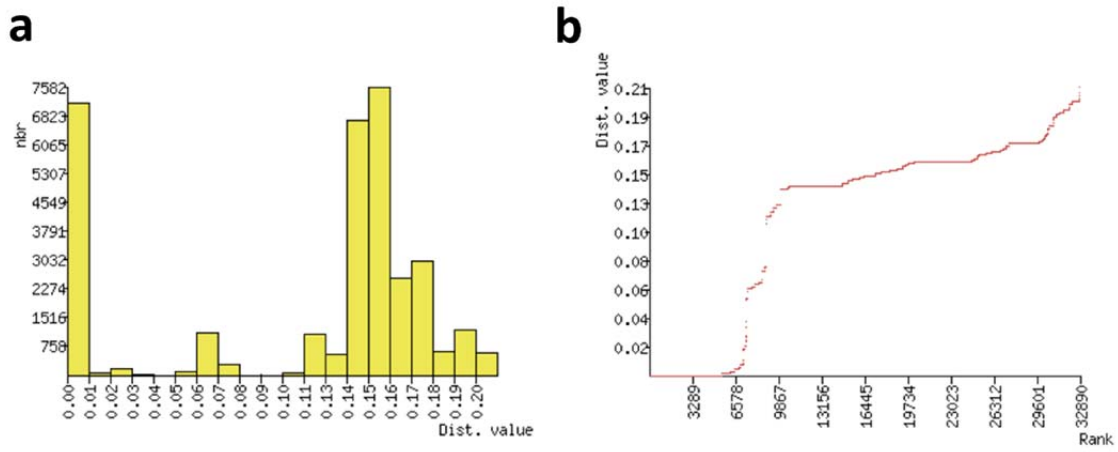


Fig. S1. ABGD results. **a**. Histogram of K2P distances (nbr = number of ranks). **b**. Ranked distances.

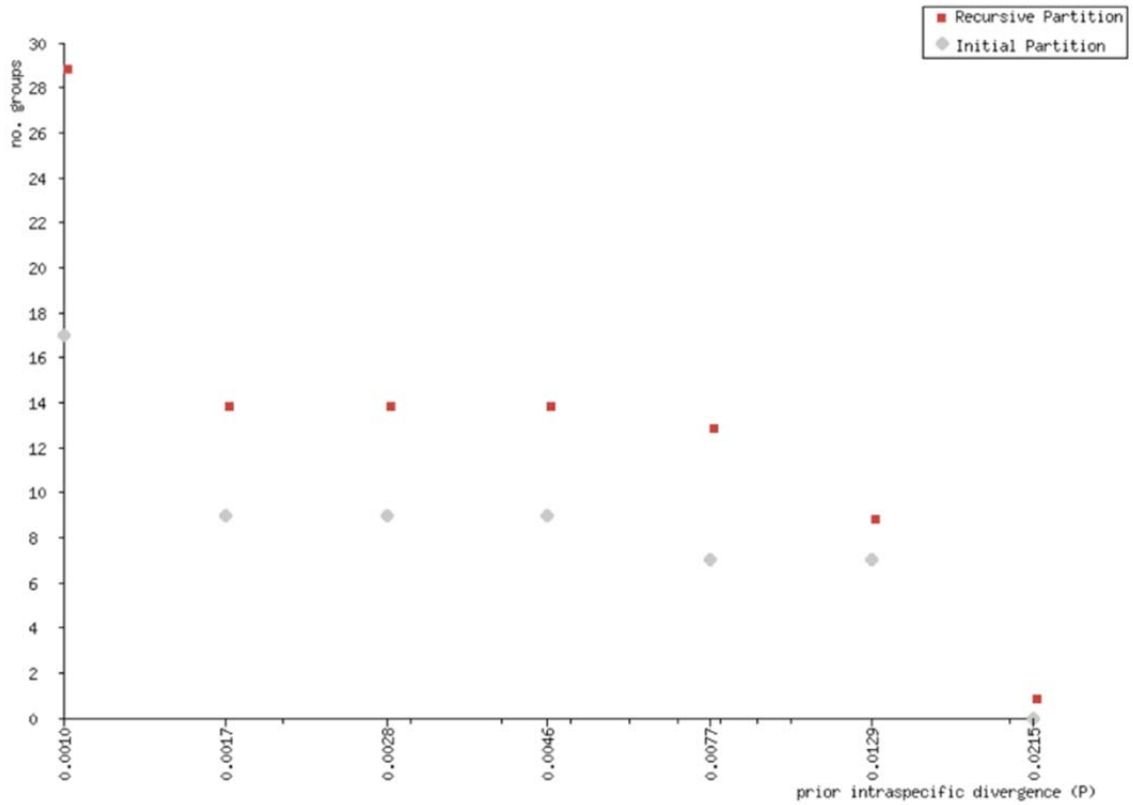


Fig. S2. ABGD results. Number of groups (PSHs) delineated by the initial and recursive partition using a range of prior intraspecific divergences (*P*).

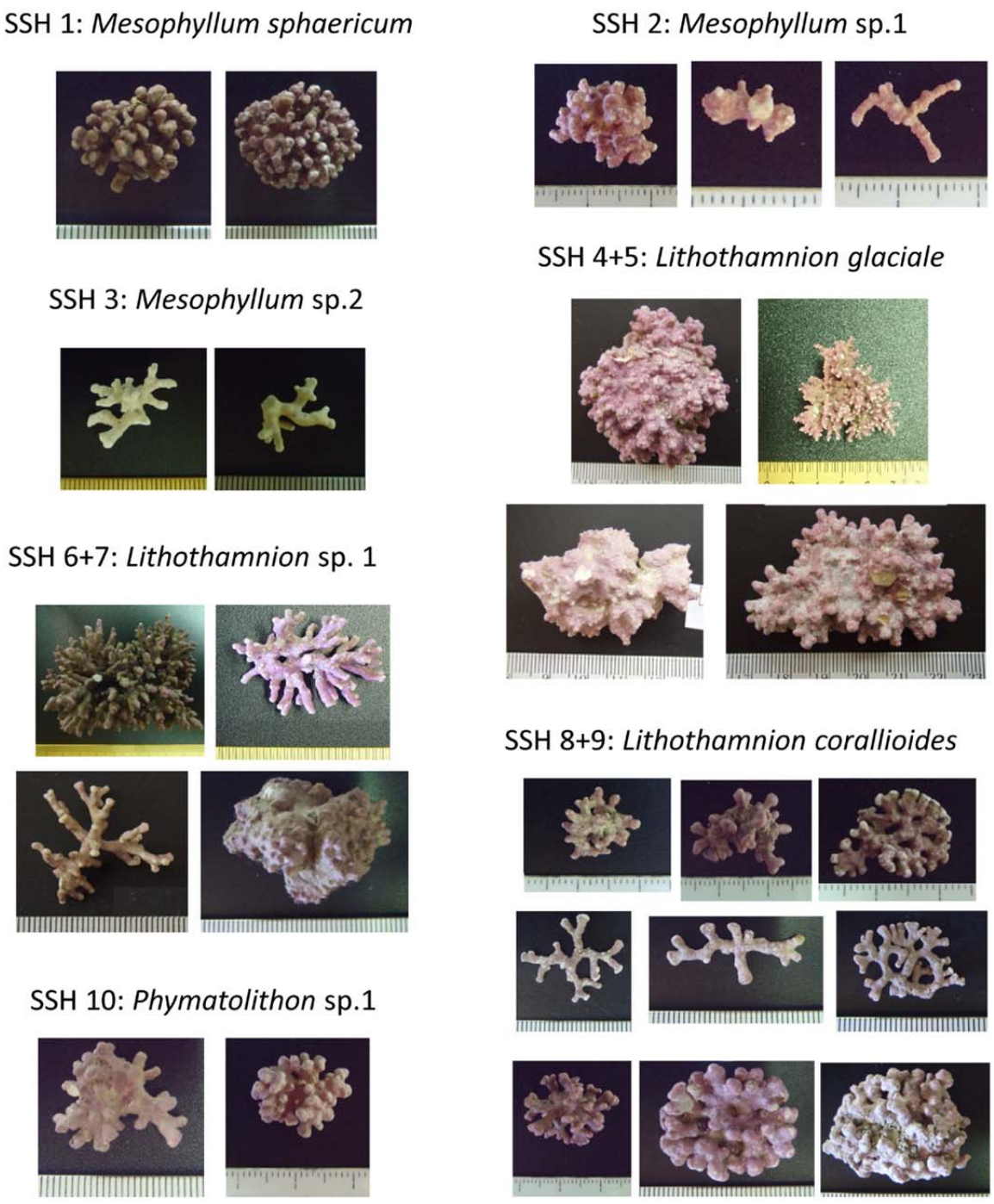


Fig. S3a. Morphological variability of Secondary Species Hypotheses (SSHs) delimited along the OSPAR area.

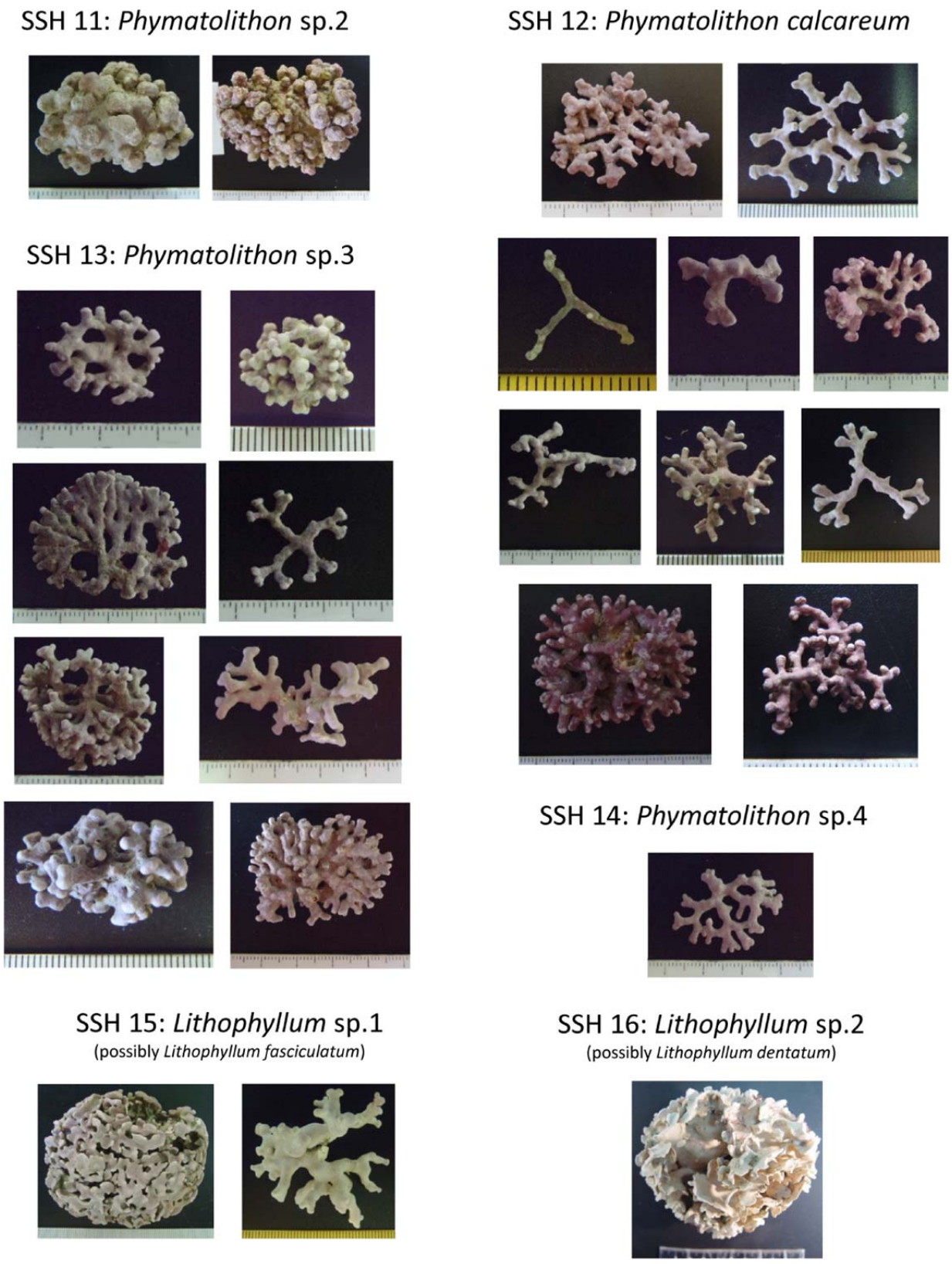


Fig. S3b. Morphological variability of Secondary Species Hypotheses (SSHs) delimited along the OSPAR area.

Table S1. Collection details with BOLD Process IDs and GenBank accession numbers for samples used in this study.

Taxon	Voucher[1]	Collectors[2]	Date	Haplotype	Country	Lat	Long	Depth (m)	BOLD Process IDs[3, 4]	GenBank accession no.[5]
Lithophyllum fasciculatum	CPVP-965	JG	01-Apr-11	COI-5P hap_16 / *psb*A hap_5	France (Brittany)	48.327	-4.319	1	**MAERL096-11**	KC861444 (KC819267)
	CPVP-303	JHS	22-Feb-99	COI-5P ap_16 / *psb*A hap_5	Ireland (Galway)	53.471	-10.117	3	**MAERL171-13**	KC861445 (KC819247)
Lithophyllum dentatum	CPVP-302	JHS	22-Feb-99	COI-5P hap_18 / *psb*A hap_4	Ireland (Galway)	53.471	-10.117	3	**MAERL213-13**	KC861446 (KC819246)
Lithothamnion corallioides	CPVP-802	JG	08-Mar-11	COI-5P hap_4	France (Brittany)	48.207	-4.452	10	MAERL017-11	KC861447
	CPVP-817	JG	06-Mar-11	COI-5P hap_3 / *psb*A hap_12	France (Brittany)	47.331	-3.132	5	**MAERL097-11**	KC861448 (KC819265)
	CPVP-803	JG	08-Mar-11	COI-5P hap_3	France (Brittany)	48.212	-4.455	10	MAERL181-13	KC861449
	CPVP-807*	JG	08-Mar-11	COI-5P hap_3	France (Brittany)	48.293	-4.579	15	MAERL179-13	KC861451
	CPVP-808*	JG	08-Mar-11	COI-5P hap_3 / *psb*A hap_12	France (Brittany)	48.293	-4.579	15	**MAERL178-13**	KC861452 (KC819264)
	CPVP-813*	JG	08-Mar-11	COI-5P hap_3	France (Brittany)	48.293	-4.579	15	MAERL027-11	KC861465
	CPVP-820	JG	06-Mar-11	COI-5P hap_3	France (Brittany)	47.331	-3.132	5	MAERL177-13	KC861453
	CPVP-823	JG	06-Mar-11	COI-5P hap_3	France (Brittany)	47.331	-3.132	5	MAERL176-13	KC861454
	CPVP-825	JG	06-Mar-11	COI-5P hap_3	France (Brittany)	47.331	-3.132	5	MAERL175-13	KC861455
	CPVP-819	JG	06-Mar-11	COI-5P hap_3	France (Brittany)	47.331	-3.132	5	MAERL030-11	KC861462
	CPVP-816	JG	06-Mar-11	COI-5P hap_3	France (Brittany)	47.331	-3.132	5	MAERL029-11	KC861463
	CPVP-818	JG	06-Mar-11	COI-5P hap_3	France (Brittany)	47.331	-3.132	5	MAERL028-11	KC861464
	CPVP-801	JG	08-Mar-11	COI-5P hap_3	France (Brittany)	48.207	-4.452	10	MAERL021-11	KC861471
	CPVP-799	JG	08-Mar-11	COI-5P hap_3	France (Brittany)	48.212	-4.455	10	MAERL183-13	KC861502
	CPVP-794	JG	08-Mar-11	COI-5P hap_3	France (Brittany)	48.212	-4.455	10	MAERL184-13	KC861501

CPVP-1238	JJ	22-Jul-11	COI-5P hap_4	France (La Rochelle)	46.233	-1.381	12	MAERL188-13	KC861497
CPVP-1227	JJ	22-Jul-11	COI-5P hap_3	France (La Rochelle)	46.233	-1.381	12	MAERL187-13	KC861498
CPVP-1231	JJ	22-Jul-11	COI-5P hap_4	France (La Rochelle)	46.233	-1.381	12	MAERL186-13	KC861499
CPVP-1232	JJ	22-Jul-11	COI-5P hap_4	France (La Rochelle)	46.233	-1.381	12	MAERL185-13	KC861500
CPVP-166	IB, RB, CP, VP	23-Nov-10	COI-5P hap_3	Spain (Galicia)	42.561	-8.890	6	MAERL180-13	KC861450
CPVP-1077	IB, FB, VP	23-Jun-11	COI-5P hap_3	Spain (Galicia)	42.569	-8.890	4	MAERL174-13	KC861456
CPVP-1079	IB, FB, VP	23-Jun-11	COI-5P hap_3	Spain (Galicia)	42.569	-8.890	4	MAERL173-13	KC861457
CPVP-649	IB, RB, VP	05-Apr-11	COI-5P hap_3	Spain (Galicia)	42.212	-8.896	11	MAERL034-11	KC861458
CPVP-564	IB, RB, CP, VP	31-Mar-11	COI-5P hap_3	Spain (Galicia)	42.788	-9.019	11	MAERL033-11	KC861459
CPVP-563	IB, RB, CP, VP	31-Mar-11	COI-5P hap_3 / *psb*A hap_12	Spain (Galicia)	42.788	-9.019	11	**MAERL032-11**	KC861460 (KC819256)
CPVP-561	IB, RB, CP, VP	31-Mar-11	COI-5P hap_3	Spain (Galicia)	42.788	-9.019	11	MAERL031-11	KC861461
CPVP-684	IB, RB, VP	05-Apr-11	COI-5P hap_3	Spain (Galicia)	42.258	-8.751	5	MAERL026-11	KC861466
CPVP-691	IB, RB, VP	05-Apr-11	COI-5P hap_3 / *psb*A hap_12	Spain (Galicia)	42.258	-8.751	5	**MAERL025-11**	KC861467 (KC819261)
CPVP-697	IB, RB, VP	05-Apr-11	COI-5P hap_3	Spain (Galicia)	42.258	-8.751	5	MAERL024-11	KC861468
CPVP-677	IB, RB, VP	05-Apr-11	COI-5P hap_3	Spain (Galicia)	42.258	-8.751	5	MAERL023-11	KC861469
CPVP-699	IB, RB, VP	05-Apr-11	COI-5P hap_3	Spain (Galicia)	42.258	-8.751	5	MAERL022-11	KC861470
CPVP-138	IB, RB, CP, VP	23-Nov-10	COI-5P hap_3	Spain (Galicia)	42.569	-8.890	4	MAERL018-11	KC861472
CPVP-631	IB, RB, VP	07-Apr-11	COI-5P hap_3	Spain (Galicia)	42.341	-8.810	9	MAERL211-13	KC861474
CPVP-1250	LK, DP	25-Jul-11	COI-5P hap_4	UK (Northern Ireland)	54.378	-5.565	10	MAERL212-13	KC861473
CPVP-1164	FB	14-Jul-11	COI-5P hap_3	UK (Wales)	51.709	-5.085	4	MAERL210-13	KC861475
CPVP-1184	FB	14-Jul-11	COI-5P hap_3	UK (Wales)	51.709	-5.085	4	MAERL209-13	KC861476
CPVP-1189	FB	14-Jul-11	COI-5P hap_3	UK (Wales)	51.709	-5.085	4	MAERL208-13	KC861477

	CPVP-1191	FB	14-Jul-11	COI-5P hap_3	UK (Wales)	51.709	-5.085	4	MAERL207-13	KC861478
	CPVP-1192	FB	14-Jul-11	COI-5P hap_3	UK (Wales)	51.709	-5.085	4	MAERL206-13	KC861479
	CPVP-1193	FB	14-Jul-11	COI-5P hap_3	UK (Wales)	51.709	-5.085	4	MAERL205-13	KC861480
	CPVP-1196	FB	14-Jul-11	COI-5P hap_3	UK (Wales)	51.709	-5.085	4	MAERL204-13	KC861481
	CPVP-1199	FB	14-Jul-11	COI-5P hap_3	UK (Wales)	51.709	-5.085	4	MAERL203-13	KC861482
	CPVP-1201	FB	14-Jul-11	COI-5P hap_3	UK (Wales)	51.709	-5.085	4	MAERL202-13	KC861483
	CPVP-1163	FB	14-Jul-11	COI-5P hap_3	UK (Wales)	51.709	-5.085	4	MAERL201-13	KC861484
	CPVP-1165	FB	14-Jul-11	COI-5P hap_3	UK (Wales)	51.709	-5.085	4	MAERL200-13	KC861485
	CPVP-1166	FB	14-Jul-11	COI-5P hap_3	UK (Wales)	51.709	-5.085	4	MAERL199-13	KC861486
	CPVP-1167	FB	14-Jul-11	COI-5P hap_25	UK (Wales)	51.709	-5.085	4	MAERL198-13	KC861487
	CPVP-1171	FB	14-Jul-11	COI-5P hap_3	UK (Wales)	51.709	-5.085	4	MAERL197-13	KC861488
	CPVP-1172	FB	14-Jul-11	COI-5P hap_3	UK (Wales)	51.709	-5.085	4	MAERL196-13	KC861489
	CPVP-1173	FB	14-Jul-11	COI-5P hap_3	UK (Wales)	51.709	-5.085	4	MAERL195-13	KC861490
	CPVP-1174	FB	14-Jul-11	COI-5P hap_3	UK (Wales)	51.709	-5.085	4	MAERL194-13	KC861491
	CPVP-1176	FB	14-Jul-11	COI-5P hap_3	UK (Wales)	51.709	-5.085	4	MAERL193-13	KC861492
	CPVP-1179	FB	14-Jul-11	COI-5P hap_3	UK (Wales)	51.709	-5.085	4	MAERL192-13	KC861493
	CPVP-1181	FB	14-Jul-11	COI-5P hap_3	UK (Wales)	51.709	-5.085	4	MAERL191-13	KC861494
	CPVP-1182	FB	14-Jul-11	COI-5P hap_3	UK (Wales)	51.709	-5.085	4	MAERL190-13	KC861495
	CPVP-1183	FB	14-Jul-11	COI-5P hap_3	UK (Wales)	51.709	-5.085	4	MAERL189-13	KC861496
Lithothamnion glaciale	CPVP-93	VH	01-May-08	COI-5P hap_7	Norway (Skarsundet)	60.143	5.166	11	MAERL229-13	KC861503
	CPVP-1401	VH	01-May-08	COI-5P hap_7	Norway (Skarsundet)	60.143	5.166	11	MAERL228-13	KC861504
	CPVP-1403	VH	01-May-08	COI-5P hap_7	Norway (Skarsundet)	60.143	5.166	11	MAERL227-13	KC861505
	CPVP-91	VH	01-May-08	COI-5P hap_7 / *psb*A hap_2	Norway (Skarsundet)	60.143	5.166	11	**MAERL230-13**	KC861508 (KC819244)
	CPVP-1448*	JB	01-Jun-10	COI-5P hap_7	Norway (Svalbard)	78.955	9.668	10	MAERL226-13	KC861506

	CPVP-1443*	JB	01-Jun-10	COI-5P hap_24 / *psb*A hap_15	Norway (Svalbard)	78.955	9.668	10	**MAERL225-13**	KC861507 (KC819270)
	CPVP-1444*	JB	01-Jun-10	COI-5P hap_7 / *psb*A hap_15	Norway (Svalbard)	78.955	9.668	10	**MAERL224-13**	KC861509 (KC819271)
Lithothamnion sp. 1	CPVP-30	VP, HH, ARE	20-Jul-04	COI-5P hap_17	Iceland (Hvalfjoerdur)	64.360	-21.753	4	MAERL218-13	KC861510
	CPVP-28	VP, HH, ARE	20-Jul-04	COI-5P hap_17	Iceland (Hvalfjoerdur)	64.360	-21.753	4	MAERL219-13	KC861515
	CPVP-29	VP, HH, ARE	20-Jul-04	COI-5P hap_19	Iceland (Hvalfjordur)	64.360	-21.753	3	MAERL035-11	KC861516
	CPVP-92	VH	01-May-08	COI-5P hap_5 / *psb*A hap_3	Norway (Skarsundet)	60.143	5.166	11	**MAERL222-13**	KC861512 (KC819245)
	CPVP-1451	JR	01-Jan-02	COI-5P hap_5	Norway (Hordaland)	60.541	4.846	7	MAERL221-13	KC861513
	CPVP-1240	LK, DP	25-Jul-11	COI-5P hap_17	UK (Northern Ireland)	54.378	-5.565	10	MAERL223-13	KC861511
	CPVP-306	NK	29-Oct-10	COI-5P hap_17	UK (Scotland)	56.022	-5.611	7	MAERL220-13	KC861514
	CPVP-305	NK	29-Oct-10	COI-5P hap_17 / *psb*A hap_3	UK (Scotland)	56.022	-5.611	7	**MAERL011-11**	KC861517 (KC819248)
Mesophyllum sp. 1	CPVP-514	PN, MR	23-Feb-11	COI-5P hap_11	Portugal (Algarve)	37.046	-8.336	20	MAERL014-11	KC861518
	CPVP-464	PN, MR	02-Mar-11	COI-5P hap_11 / *psb*A hap_8	Portugal (Algarve)	37.027	-8.317	20	**MAERL019-11**	KC861519 (KC819252)
	CPVP-465	PN, MR	02-Mar-11	COI-5P hap_11	Portugal (Algarve)	37.027	-8.317	20	MAERL013-11	KC861520
	CPVP-467	PN, MR	02-Mar-11	COI-5P hap_11	Portugal (Algarve)	37.027	-8.317	20	MAERL020-11	KC861521
Mesophyllum sp. 2	CPVP-1157	CS	15-Apr-10	COI-5P hap_27 / *psb*A hap_14	Spain (Canary Islands)	28.642	-17.723	15	**MAERL217-13**	KC861522 (KC819269)
	CPVP-307	CS	15-Apr-10	COI-5P hap_27 / *psb*A hap_6	Spain (Canary Islands)	28.642	-17.723	15	**MAERL016-11**	KC861523 (KC819249)
Mesophyllum sphaericum	**CPVP-1084***	IB, FB, VP	23-Jun-11	COI-5P hap_2	Spain (Galicia)	42.600	-8.874	3	MAERL216-13	KC861524
	CPVP-1130*	IB, FB, VP	23-Jun-11	COI-5P hap_2 / *psb*A hap_13	Spain (Galicia)	42.600	-8.874	3	**MAERL214-13**	KC861525 (KC819268)

	CPVP-776**	IB, VP	14-Oct-08	COI-5P hap_2 / *psb*A hap_13	Spain (Galicia)	42.600	-8.874	3	**MAERL015-11**	KC861526 (KC819262)
	CPVP-1115*	IB, FB, VP	23-Jun-11	COI-5P hap_6	Spain (Galicia)	42.600	-8.874	3	MAERL215-13	KC861527
Phymatolithon calcareum	CPVP-555	IB, RB, CP, VP	31-Mar-11	COI-5P hap_26	Spain (Galicia)	42.788	-9.019	11	MAERL041-11	KC861528
	CPVP-753	VP, IB, RB	05-Apr-11	COI-5P hap_26	Spain (Galicia)	42.227	-8.777	11	MAERL136-13	KC861546
	CPVP-603	IB, RB, VP	07-Apr-11	COI-5P hap_26	Spain (Galicia)	42.394	-8.915	13	MAERL132-13	KC861550
	CPVP-607	IB, RB, VP	07-Apr-11	COI-5P hap_28	Spain (Galicia)	42.394	-8.915	13	MAERL131-13	KC861551
	CPVP-615	IB, RB, VP	07-Apr-11	COI-5P hap_26	Spain (Galicia)	42.394	-8.915	13	MAERL130-13	KC861552
	CPVP-162	IB, RB, CP, VP	23-Nov-10	COI-5P hap_26	Spain (Galicia)	42.561	-8.890	6	MAERL117-13	KC861565
	CPVP-163	IB, RB, CP, VP	23-Nov-10	COI-5P hap_26	Spain (Galicia)	42.561	-8.890	6	MAERL116-13	KC861566
	CPVP-164	IB, RB, CP, VP	23-Nov-10	COI-5P hap_26	Spain (Galicia)	42.561	-8.890	6	MAERL115-13	KC861567
	CPVP-170	IB, RB, CP, VP	23-Nov-10	COI-5P hap_26	Spain (Galicia)	42.561	-8.890	6	MAERL114-13	KC861568
	CPVP-655	IB, RB, VP	05-Apr-11	COI-5P hap_26	Spain (Galicia)	42.212	-8.896	11	MAERL113-13	KC861569
	CPVP-1103	IB, FB, VP	23-Jun-11	COI-5P hap_26	Spain (Galicia)	42.600	-8.874	3	MAERL112-13	KC861570
	CPVP-1104	IB, FB, VP	23-Jun-11	COI-5P hap_26	Spain (Galicia)	42.600	-8.874	3	MAERL111-13	KC861571
	CPVP-1108	IB, FB, VP	23-Jun-11	COI-5P hap_26	Spain (Galicia)	42.600	-8.874	3	MAERL110-13	KC861572
	CPVP-1109	IB, FB, VP	23-Jun-11	COI-5P hap_26	Spain (Galicia)	42.600	-8.874	3	MAERL109-13	KC861573
	CPVP-1110	IB, FB, VP	23-Jun-11	COI-5P hap_26	Spain (Galicia)	42.600	-8.874	3	MAERL108-13	KC861574
	CPVP-1118	IB, FB, VP	23-Jun-11	COI-5P hap_26	Spain (Galicia)	42.600	-8.874	3	MAERL107-13	KC861575
	CPVP-1119	IB, FB, VP	23-Jun-11	COI-5P hap_26	Spain (Galicia)	42.600	-8.874	3	MAERL106-13	KC861576
	CPVP-1120	IB, FB, VP	23-Jun-11	COI-5P hap_26	Spain (Galicia)	42.600	-8.874	3	MAERL105-13	KC861577
	CPVP-1121	IB, FB, VP	23-Jun-11	COI-5P hap_26	Spain (Galicia)	42.600	-8.874	3	MAERL104-13	KC861578
	CPVP-1129	IB, FB, VP	23-Jun-11	COI-5P hap_26	Spain (Galicia)	42.600	-8.874	3	MAERL103-13	KC861579
	CPVP-1065	IB, FB, VP	23-Jun-11	COI-5P hap_26	Spain (Galicia)	42.569	-8.890	5	MAERL102-13	KC861580
	CPVP-1067	IB, FB, VP	23-Jun-11	COI-5P hap_26	Spain (Galicia)	42.569	-8.890	5	MAERL101-13	KC861581
	CPVP-1078	IB, FB, VP	13-Jun-11	COI-5P hap_26	Spain (Galicia)	42.569	-8.890	5	MAERL100-13	KC861582

CPVP-174	IB, RB, CP, VP	23-Nov-10	COI-5P hap_26	Spain (Galicia)	42.569	-8.890	4	MAERL099-13	KC861583
CPVP-207	IB, RB, CP, VP	23-Nov-10	COI-5P hap_26	Spain (Galicia)	42.569	-8.890	4	MAERL098-13	KC861584
CPVP-628	IB, RB, VP	07-Apr-11	COI-5P hap_26	Spain (Galicia)	42.341	-8.810	9	MAERL036-11	KC861586
CPVP-629	IB, RB, VP	07-Apr-11	COI-5P hap_26	Spain (Galicia)	42.341	-8.810	9	MAERL037-11	KC861587
CPVP-558	IB, RB, CP, VP	31-Mar-11	COI-5P hap_26	Spain (Galicia)	42.788	-9.019	11	MAERL040-11	KC861590
CPVP-560	IB, RB, CP, VP	31-Mar-11	COI-5P hap_26	Spain (Galicia)	42.788	-9.019	11	MAERL042-11	KC861592
CPVP-554	IB, RB, CP, VP	31-Mar-11	COI-5P hap_26	Spain (Galicia)	42.788	-9.019	11	MAERL043-11	KC861593
CPVP-310	IB, VP	12-Jun-09	COI-5P hap_26	Spain (Galicia)	42.788	-9.019	11	MAERL044-11	KC861594
CPVP-566	IB, RB, CP, VP	31-Mar-11	COI-5P hap_26 / *psb*A hap_1	Spain (Galicia)	42.788	-9.019	11	**MAERL045-11**	KC861595 (KC819257)
CPVP-565	IB, RB, CP, VP	31-Mar-11	COI-5P hap_26	Spain (Galicia)	42.788	-9.019	11	MAERL046-11	KC861596
CPVP-766	IB, RB, VP	07-Apr-11	COI-5P hap_26	Spain (Galicia)	42.394	-8.915	13	MAERL047-11	KC861597
CPVP-167	IB, RB, CP, VP	23-Nov-10	COI-5P hap_26	Spain (Galicia)	42.561	-8.890	6	MAERL048-11	KC861598
CPVP-665	IB, RB, VP	05-Apr-11	COI-5P hap_26 / *psb*A hap_1	Spain (Galicia)	42.212	-8.896	11	**MAERL049-11**	KC861599 (KC819259)
CPVP-648	IB, RB, VP	05-Apr-11	COI-5P hap_26	Spain (Galicia)	42.212	-8.896	11	MAERL050-11	KC861600
CPVP-654	IB, RB, VP	05-Apr-11	COI-5P hap_26	Spain (Galicia)	42.212	-8.896	11	MAERL051-11	KC861601
CPVP-696	IB, RB, VP	05-Apr-11	COI-5P hap_26	Spain (Galicia)	42.258	-8.751	5	MAERL053-11	KC861603
CPVP-275	IB, RB, CP, VP	23-Nov-10	COI-5P hap_26	Spain (Galicia)	42.569	-8.890	4	MAERL061-11	KC861611
CPVP-130	IB, RB, CP, VP	23-Nov-10	COI-5P hap_26	Spain (Galicia)	42.569	-8.890	4	MAERL062-11	KC861612
CPVP-135	IB, RB, CP, VP	23-Nov-10	COI-5P hap_26	Spain (Galicia)	42.569	-8.890	4	MAERL063-11	KC861613
CPVP-151	IB, RB, CP, VP	23-Nov-10	COI-5P hap_26	Spain (Galicia)	42.569	-8.890	4	MAERL064-11	KC861614
CPVP-157	IB, RB, CP, VP	23-Nov-10	COI-5P hap_26	Spain (Galicia)	42.569	-8.890	4	MAERL065-11	KC861615
CPVP-1450	JR	01-Jan-02	COI-5P hap_26	Norway (Hordaland)	60.541	4.846	7	MAERL133-13	KC861549
CPVP-1242	LK, DP	25-Jul-11	COI-5P hap_26	UK (Northern Ireland)	54.378	-5.565	10	MAERL149-13	KC861533
CPVP-1243	LK, DP	25-Jul-11	COI-5P hap_26	UK (Northern Ireland)	54.378	-5.565	10	MAERL148-13	KC861534

CPVP-1244	LK, DP	25-Jul-11	COI-5P hap_26	UK (Northern Ireland)	54.378	-5.565	10	MAERL147-13	KC861535
CPVP-1245	LK, DP	25-Jul-11	COI-5P hap_26	UK (Northern Ireland)	54.378	-5.565	10	MAERL146-13	KC861536
CPVP-1246	LK, DP	25-Jul-11	COI-5P hap_26	UK (Northern Ireland)	54.378	-5.565	10	MAERL145-13	KC861537
CPVP-1247	LK, DP	25-Jul-11	COI-5P hap_26	UK (Northern Ireland)	54.378	-5.565	10	MAERL144-13	KC861538
CPVP-1251	LK, DP	25-Jul-11	COI-5P hap_26	UK (Northern Ireland)	54.378	-5.565	10	MAERL143-13	KC861539
CPVP-1252	LK, DP	25-Jul-11	COI-5P hap_26	UK (Northern Ireland)	54.378	-5.565	10	MAERL142-13	KC861540
CPVP-1253	LK, DP	25-Jul-11	COI-5P hap_26	UK (Northern Ireland)	54.378	-5.565	10	MAERL141-13	KC861541
CPVP-1254	LK, DP	25-Jul-11	COI-5P hap_26	UK (Northern Ireland)	54.378	-5.565	10	MAERL140-13	KC861542
CPVP-43	KC, VP	07-May-05	COI-5P hap_29	UK (England)	50.605	-1.868	12	MAERL135-13	KC861547
CPVP-44	KC, VP	07-May-05	COI-5P hap_26	UK (England)	50.605	-1.868	12	MAERL134-13	KC861548
CPVP-46*	JHS	02-Jun-10	COI-5P hap_26	UK (England)	50.164	-5.022	18	MAERL118-13	KC861564
CPVP-48*	JHS	02-Jun-10	COI-5P hap_26	UK (England)	50.164	-5.022	18	MAERL012-11	KC861585
CPVP-47*	JHS	02-Jun-10	COI-5P hap_26 / *psb*A hap_1	UK (England)	50.164	-5.022	18	**MAERL054-11**	KC861604 (KC819243)
CPVP-1187	FB	14-Jul-11	COI-5P hap_26	UK (Wales)	51.709	-5.085	4	MAERL126-13	KC861556
CPVP-1195	FB	14-Jul-11	COI-5P hap_26	UK (Wales)	51.709	-5.085	4	MAERL125-13	KC861557
CPVP-1188	FB	14-Jul-11	COI-5P hap_26	UK (Wales)	51.709	-5.085	4	MAERL124-13	KC861558
CPVP-780	MM, JHK	21-Oct-10	COI-5P hap_26	Ireland (Galway)	53.246	-9.628	5	MAERL120-13	KC861562
CPVP-783	MM, JHK	21-Oct-10	COI-5P hap_26 / *psb*A hap_1	Ireland (Galway)	53.246	-9.628	5	**MAERL119-13**	KC861563 (KC819263)
CPVP-781	MM, JHK	21-Oct-10	COI-5P hap_26	Ireland (Galway)	53.246	-9.628	5	MAERL066-11	KC861616
CPVP-778	MM	01-Jun-10	COI-5P hap_26	Ireland (Kerry)	51.809	-9.948	10	MAERL121-13	KC861561
CPVP-779	MM	01-Jun-10	COI-5P hap_26	Ireland (Kerry)	51.801	-9.940	10	MAERL052-11	KC861602
CPVP-910	TW, YF	10-May-11	COI-5P hap_26	France (Brittany)	48.711	-3.951	11	MAERL234-13	KC861530
CPVP-901	TW, YF	10-May-11	COI-5P hap_26	France (Brittany)	48.711	-3.951	11	MAERL233-13	KC861531
CPVP-900	TW, YF	10-May-11	COI-5P hap_26	France (Brittany)	48.711	-3.951	11	MAERL182-13	KC861532

	CPVP-920	TW, YF	13-May-11	COI-5P hap_26	France (Brittany)	47.720	-4.032	15	MAERL139-13	KC861543
	CPVP-921	TW, YF	13-May-11	COI-5P hap_26	France (Brittany)	47.720	-4.032	15	MAERL138-13	KC861544
	CPVP-929	TW, YF	13-May-11	COI-5P hap_26	France (Brittany)	47.720	-4.032	15	MAERL137-13	KC861545
	CPVP-858	JG, VP	10-Mar-11	COI-5P hap_26	France (Brittany)	48.386	-4.854	10	MAERL129-13	KC861553
	CPVP-959	JG, VP	13-May-11	COI-5P hap_26	France (Brittany)	48.386	-4.854	10	MAERL128-13	KC861554
	CPVP-961	JG, VP	13-May-11	COI-5P hap_26	France (Brittany)	48.386	-4.854	13	MAERL127-13	KC861555
	CPVP-956	JG, VP	13-May-11	COI-5P hap_26	France (Brittany)	48.386	-4.854	10	MAERL038-11	KC861588
	CPVP-916	TW, YF	13-May-11	COI-5P hap_26	France (Brittany)	47.720	-4.032	15	MAERL039-11	KC861589
	CPVP-912	TW, YF	10-May-11	COI-5P hap_26 / *psb*A hap_1	France (Brittany)	48.711	-3.951	11	**MAERL055-11**	KC861605 (KC819266)
	CPVP-909	TW, YF	10-May-11	COI-5P hap_26	France (Brittany)	48.711	-3.951	11	MAERL056-11	KC861606
	CPVP-903	TW, YF	10-May-11	COI-5P hap_26	France (Brittany)	48.711	-3.951	11	MAERL057-11	KC861607
	CPVP-906	TW, YF	10-May-11	COI-5P hap_26	France (Brittany)	48.711	-3.951	11	MAERL058-11	KC861608
	CPVP-897	TW, YF	10-May-11	COI-5P hap_26	France (Brittany)	48.711	-3.951	11	MAERL059-11	KC861609
	CPVP-899	TW, YF	10-May-11	COI-5P hap_26	France (Brittany)	48.711	-3.951	11	MAERL060-11	KC861610
	CPVP-1234	JJ	22-Jul-11	COI-5P hap_26	France (La Rochelle)	46.233	-1.381	12	MAERL123-13	KC861559
	CPVP-1236	JJ	22-Jul-11	COI-5P hap_26	France (La Rochelle)	46.233	-1.381	12	MAERL122-13	KC861560
	BM000712373**	WF	11-Dec-83	COI-5P hap_26	UK (England)	50.16	-5.02		MAERL237-13	KF808323
Phymatolithon sp. 1	CPVP-868	VP	11-Mar-11	COI-5P hap_15	France (Brittany)	48.790	-2.883	30	MAERL172-13	KC861663
	CPVP-510	PN, MR	23-Feb-11	COI-5P hap_15 / *psb*A hap_11	Portugal (Algarve)	37.046	-8.336	20	**MAERL069-11**	KC861664 (KC819255)
Phymatolithon sp. 2	CPVP-443	PW	04-Apr-11	COI-5P hap_23 / *psb*A hap_7	Portugal (Madeira)	32.641	-16.829	18	**MAERL068-11**	KC861665 (KC819251)
	CPVP-439	PW	04-Apr-11	COI-5P hap_20	Portugal (Madeira)	32.641	-16.829	18	MAERL232-13	KC861666
	CPVP-440	PW	04-Apr-11	COI-5P hap_21	Portugal (Madeira)	32.641	-16.829	18	MAERL231-13	KC861667

	CPVP-441	PW	04-Apr-11	COI-5P hap_22 / *psb*A hap_7	Portugal (Madeira)	32.641	-16.829	18	**MAERL067-11**	KC861668 (KC819250)
Phymatolithon sp. 3	CPVP-627	IB, RB, VP	07-Apr-11	COI-5P hap_1	Spain (Galicia)	42.341	-8.810	9	MAERL168-13	KC861617
	CPVP-49	IB, VP	19-May-09	COI-5P hap_1	Spain (Galicia)	42.227	-8.777	11	MAERL078-11	KC861619
	CPVP-689	IB, RB, VP	05-Apr-11	COI-5P hap_1	Spain (Galicia)	42.258	-8.751	5	MAERL079-11	KC861620
	CPVP-685	IB, RB, VP	05-Apr-11	COI-5P hap_1	Spain (Galicia)	42.258	-8.751	5	MAERL080-11	KC861621
	CPVP-686	IB, RB, VP	05-Apr-11	COI-5P hap_1	Spain (Galicia)	42.258	-8.751	5	MAERL081-11	KC861622
	CPVP-681	IB, RB, VP	05-Apr-11	COI-5P hap_1	Spain (Galicia)	42.258	-8.751	5	MAERL082-11	KC861623
	CPVP-680	IB, RB, VP	05-Apr-11	COI-5P hap_1	Spain (Galicia)	42.258	-8.751	5	MAERL083-11	KC861624
	CPVP-695	IB, RB, VP	05-Apr-11	COI-5P hap_1	Spain (Galicia)	42.258	-8.751	5	MAERL084-11	KC861625
	CPVP-694	IB, RB, VP	05-Apr-11	COI-5P hap_1	Spain (Galicia)	42.258	-8.751	5	MAERL085-11	KC861626
	CPVP-676	IB, RB, VP	05-Apr-11	COI-5P hap_1 / *psb*A hap_9	Spain (Galicia)	42.258	-8.751	5	**MAERL086-11**	KC861627 (KC819260)
	CPVP-622	IB, RB, VP	07-Apr-11	COI-5P hap_1	Spain (Galicia)	42.341	-8.810	9	MAERL170-13	KC861628
	CPVP-644	IB, RB, VP	05-Apr-11	COI-5P hap_1	Spain (Galicia)	42.212	-8.896	11	MAERL088-11	KC861635
	CPVP-645	IB, RB, VP	05-Apr-11	COI-5P hap_1	Spain (Galicia)	42.212	-8.896	11	MAERL089-11	KC861636
	CPVP-646	IB, RB, VP	05-Apr-11	COI-5P hap_1	Spain (Galicia)	42.212	-8.896	11	MAERL090-11	KC861637
	CPVP-670	IB, RB, VP	05-Apr-11	COI-5P hap_1	Spain (Galicia)	42.212	-8.896	11	MAERL091-11	KC861638
	CPVP-664	IB, RB, VP	05-Apr-11	COI-5P hap_1	Spain (Galicia)	42.212	-8.896	11	MAERL092-11	KC861639
	CPVP-669	IB, RB, VP	05-Apr-11	COI-5P hap_1	Spain (Galicia)	42.212	-8.896	11	MAERL093-11	KC861640
	CPVP-618	IB, RB, VP	07-Apr-11	COI-5P hap_1	Spain (Galicia)	42.341	-8.810	9	MAERL094-11	KC861641
	CPVP-639	IB, RB, VP	07-Apr-11	COI-5P hap_1 / *psb*A hap_9	Spain (Galicia)	42.341	-8.810	9	**MAERL095-11**	KC861642 (KC819258)
	CPVP-1060	IB, FB, VP	23-Jun-11	COI-5P hap_1	Spain (Galicia)	42.569	-8.890	4	MAERL151-13	KC861644
	CPVP-1136	IB, FB, VP	23-Jun-11	COI-5P hap_1	Spain (Galicia)	42.600	-8.874	3	MAERL152-13	KC861645
	CPVP-1134	IB, FB, VP	23-Jun-11	COI-5P hap_1	Spain (Galicia)	42.600	-8.874	3	MAERL153-13	KC861646

CPVP-611	IB, RB, VP	07-Apr-11	COI-5P hap_1	Spain (Galicia)	42.394	-8.915	13	MAERL154-13	KC861647
CPVP-600	IB, RB, VP	07-Apr-11	COI-5P hap_1	Spain (Galicia)	42.394	-8.915	13	MAERL155-13	KC861648
CPVP-1064	IB, FB, VP	23-Jun-11	COI-5P hap_1	Spain (Galicia)	42.569	-8.890	4	MAERL156-13	KC861649
CPVP-1261	IB, VG, VP	05-Aug-11	COI-5P hap_8	Spain (Galicia)	42.491	-8.999	5	MAERL157-13	KC861650
CPVP-1260	IB, VG, VP	05-Aug-11	COI-5P hap_1	Spain (Galicia)	42.491	-8.999	5	MAERL158-13	KC861651
CPVP-1259	IB, VG, VP	05-Aug-11	COI-5P hap_1	Spain (Galicia)	42.491	-8.999	5	MAERL159-13	KC861652
CPVP-1258	IB, VG, VP	05-Aug-11	COI-5P hap_8	Spain (Galicia)	42.491	-8.999	5	MAERL160-13	KC861653
CPVP-1257	IB, VG, VP	05-Aug-11	COI-5P hap_1	Spain (Galicia)	42.491	-8.999	5	MAERL161-13	KC861654
CPVP-1256	IB, VG, VP	05-Aug-11	COI-5P hap_8	Spain (Galicia)	42.491	-8.999	5	MAERL162-13	KC861655
CPVP-749	VP, IB, RB	05-Apr-11	COI-5P hap_1	Spain (Galicia)	42.227	-8.777	11	MAERL163-13	KC861656
CPVP-748	VP, IB, RB	05-Apr-11	COI-5P hap_1	Spain (Galicia)	42.227	-8.777	11	MAERL164-13	KC861657
CPVP-51	IB, VP	19-May-09	COI-5P hap_1	Spain (Galicia)	42.227	-8.777	11	MAERL165-13	KC861658
CPVP-50	IB, VP	19-May-09	COI-5P hap_1	Spain (Galicia)	42.227	-8.777	11	MAERL166-13	KC861659
CPVP-633	IB, RB, VP	07-Apr-11	COI-5P hap_1	Spain (Galicia)	42.341	-8.810	9	MAERL167-13	KC861660
CPVP-626	IB, RB, VP	07-Apr-11	COI-5P hap_1	Spain (Galicia)	42.341	-8.810	9	MAERL169-13	KC861662
CPVP-501	PN, MR	23-Feb-11	COI-5P hap_13	Portugal (Algarve)	37.046	-8.336	20	MAERL076-11	KC861661
CPVP-452	PN, MR	02-Mar-11	COI-5P hap_10	Portugal (Algarve)	37.027	-8.317	20	MAERL077-11	KC861618
CPVP-480	PN, MR	02-Mar-11	COI-5P hap_12	Portugal (Algarve)	37.027	-8.317	20	MAERL070-11	KC861629
CPVP-478	PN, MR	02-Mar-11	COI-5P hap_12 / *psb*A hap_9	Portugal (Algarve)	37.027	-8.317	20	**MAERL071-11**	KC861630 (KC819253)
CPVP-451	PN, MR	02-Mar-11	COI-5P hap_1	Portugal (Algarve)	37.027	-8.317	20	MAERL072-11	KC861631
CPVP-453	PN, MR	02-Mar-11	COI-5P hap_1	Portugal (Algarve)	37.027	-8.317	20	MAERL073-11	KC861632
CPVP-503	PN, MR	23-Feb-11	COI-5P hap_13	Portugal (Algarve)	37.046	-8.336	20	MAERL074-11	KC861633
CPVP-479	PN, MR	02-Mar-11	COI-5P hap_13	Portugal (Algarve)	37.027	-8.317	20	MAERL075-11	KC861634
CPVP-77	IB, VP, EB, PN, RS	04-Sep-08	COI-5P hap_9	Portugal (Algarve)	37.110	-8.640	15	MAERL150-13	KC861643

Phymatolithon sp. 4	CPVP-502	PN, MR	23-Feb-11	COI-5P hap_14 / *psb*A hap_10	Portugal (Algarve)	37.046	-8.336	20	**MAERL087-11**	KC861669 (KC819254)

[1]*Collections from type/neotype localities; **holotype/neotype specimen.

[2] Abbreviations for collectors include: ARE (Alfonso Ramos-Esplá), CP (Cristina Pardo), CS (Carlos Sangil), DP (Daniel Pritchard), EB (Estíbaliz Berecíbar), FB (Francis Bunker), HH (Halldor Halldorsson), IB (Ignacio Bárbara), JB (Jan Buedenbender), JG (Jacques Grall), JHK (Jazmín Hernández-Kantún), JHS (Jason M. Hall-Spencer), JJ (Jérôme Jourde), JR (Jan Rueness), KC (Ken Collins), LK (Louise Kregting), MM (Meadhbh Moriarty), MR (Miguel Rodrigues), NK (Nick Kamenos), NN (Niamh Nolan), PN (Pedro Neves), PW (Peter Wirtz), RB (Rodolfo Barreiro), RS (Rui Santos), TW (Thomas Wilfried), VG (Verónica García), VH (Vivian Husa), VP (Viviana Peña), WF (William F. Farnham), YF (Yann Fontana).

[3] BOLD Process IDs of sequence. Detailed collection data can be acquired from BOLD Systems (project "maerl-NE Atlantic", code MAERL, http://www.boldsystems.org).

[4] Process IDs in bold and underlined indicate vouchers sequenced for both COI-5P and *psb*A markers.

[5] GenBank accession number for *psb*A fragments in parenthesis; otherwise, GenBank accession numbers refer to COI-5P sequences.

Table S2a. COI-5P sequence divergence within (diagonal) and between (below diagonal) the PSHs shown in **Fig. 3**. The number of base substitutions per site from averaging over all sequence pairs between PSHs is shown. Analyses were conducted using the K2P model (Kimura 1980). In bold, values below an arbitrary 2% cutoff. n/c = not computable.

	PSH1	**PSH2**	**PSH3**	**PSH4**	**PSH5**	**PSH6**	**PSH7**	**PSH8**	**PSH9**	**PSH10**	**PSH11**	**PSH12**	**PSH13**	**PSH14**	**PSH15**	**PSH16**
PSH1	**0.002**															
PSH2	0.144	n/c														
PSH3	0.141	0.041	n/c													
PSH4	0.168	0.169	0.167	n/c												
PSH5	0.168	0.171	0.169	**0.011**	n/c											
PSH6	0.167	0.139	0.139	0.057	0.064	n/c										
PSH7	0.166	0.141	0.141	0.056	0.063	**0.002**	**0.002**									
PSH8	0.173	0.154	0.156	0.114	0.117	0.123	0.122	**0.002**								
PSH9	0.168	0.153	0.155	0.109	0.113	0.118	0.118	**0.005**	n/c							
PSH10	0.185	0.154	0.145	0.158	0.167	0.145	0.144	0.157	0.156	n/c						
PSH11	0.179	0.141	0.141	0.147	0.156	0.138	0.141	0.145	0.145	0.028	**0.003**					
PSH12	0.192	0.159	0.159	0.146	0.154	0.143	0.142	0.154	0.149	0.081	0.068	**0.006**				
PSH13	0.156	0.160	0.153	0.151	0.157	0.142	0.141	0.165	0.158	0.135	0.133	0.137	**0.004**			
PSH14	0.165	0.169	0.157	0.148	0.154	0.146	0.145	0.162	0.155	0.143	0.141	0.145	0.022	n/c		
PSH15	0.198	0.199	0.193	0.192	0.190	0.186	0.185	0.189	0.188	0.186	0.174	0.188	0.189	0.182	n/c	
PSH16	0.180	0.193	0.184	0.188	0.184	0.184	0.183	0.179	0.178	0.182	0.167	0.186	0.185	0.173	0.036	n/c

Table S2b. COI-5P sequence divergence within (diagonal) and between (below diagonal) the SSHs shown in **Fig. 4**. The number of base substitutions per site from averaging over all sequence pairs between PSHs is shown. Analyses were conducted using the K2P model (Kimura 1980). In bold, values below an arbitrary 2% cutoff. n/c = not computable.

	SSH1	**SSH2**	**SSH3**	**SSH4+5**	**SSH6+7**	**SSH8+9**	**SSH10**	**SSH11**	**SSH12**	**SSH13**	**SSH14**	**SSH15**	**SSH16**
SSH1	**0.002**												
SSH2	0.144	n/c											
SSH3	0.141	0.041	n/c										
SSH4+5	0.168	0.170	0.168	**0.011**									
SSH6+7	0.167	0.141	0.141	0.060	**0.002**								
SSH8+9	0.171	0.154	0.156	0.114	0.121	**0.004**							
SSH10	0.185	0.154	0.145	0.162	0.144	0.157	n/c						
SSH11	0.179	0.141	0.141	0.152	0.140	0.145	0.028	**0.003**					
SSH12	0.192	0.159	0.159	0.150	0.142	0.152	0.081	0.068	**0.006**				
SSH13	0.156	0.160	0.153	0.154	0.141	0.163	0.135	0.133	0.137	**0.004**			
SSH14	0.165	0.169	0.157	0.151	0.145	0.160	0.143	0.141	0.145	0.022	n/c		
SSH15	0.198	0.199	0.193	0.191	0.185	0.188	0.186	0.174	0.188	0.189	0.182	n/c	
SSH16	0.180	0.193	0.184	0.186	0.183	0.179	0.182	0.167	0.186	0.185	0.173	0.036	n/c

Table S3. Matches with our sequences in public databases (GenBank: COI-5P and *psb*A; BOLD: COI-5P). Only hits with an identity beyond a minimum threshold are reported for GenBank (98% for COI-5P, 99% for *psb*A) while BOLD's identification engine determines those queries that provide an acceptable match. Query coverage (i.e. overlap) always was > 92%. BOLD searches were conducted against all barcode records lodged on BOLD in February 12th, 2013 (1,395,901 sequences); this unvalidated library includes records without species level identification, species represented by only one or two specimens, and species with interim taxonomy. Barcode Index Numbers (BINs) are clusters of similar sequences uploaded to BOLD that are likely to correspond to biological species.

Query	Genbank best hits					BOLD best hits			
	Accession no.	Identity	Taxonomy	Voucher	Location	BIN no. (no. collections)	Identity	Taxonomy	Location
					COI-5P				
SSH4+5 (Hap_7)	HM918812	99.2%	*Lithothamnion glaciale*	GWS007542	Canada (Newfoundland and Labrador, English Harbour Eastern Cove) (47.633N 54.87W)	BOLD: AAA6958 (39 collect.)	98.9-99.9%	*Lithothamnion glaciale*	USA (Massachusetts, Maine); Canada (New Brunswick, Manitoba, Newfoundland and Labrador, Nova Scotia)
SSH4+5 (Hap_24)	HM918812	98.8%	*Lithothamnion glaciale*	GWS007542	Canada (Newfoundland and Labrador, English Harbour Eastern Cove) (47.633N 54.87W)	BOLD: AAA6958 (39 collect.)	98.6-99.2%	*Lithothamnion glaciale*	USA (Massachusetts, Maine); Canada (New Brunswick, Manitoba, Newfoundland and Labrador, Nova Scotia)
SSH6+7 (Hap_5)	No match					BOLD: ABA9580 (2 collect.)	99.9%	Corallinac sp.29BCcrust	Canada (Bristish Columbia)
SSH6+7 (Hap17)	No match					BOLD: ABA9580 (2 collect.)	100%	Corallinac sp.29BCcrust	Canada (Bristish Columbia)
SSH6+7 (Hap19)	No match					BOLD: ABA9580 (2 collect.)	99.9%	Corallinac sp.29BCcrust	Canada (Bristish Columbia)
SSH12 (Hap26)	GQ917247	100%	Uncultured Corallinales	LBC0001*	France (47.63905N 3.4166667W)	No match			
SSH12 (Hap28)	GQ917247	99.8%	Uncultured Corallinales	LBC0001*	France (47.63905N 3.4166667W)	No match			

SSH12 (Hap29)	GQ917247	99.2%	Uncultured Corallinales	LBC0001*	France (47.63905N 3.4166667W)	No match
SSH16 (Hap18)	GQ917510	99.9%	Uncultured Corallinales	LBC004	France (47.63905N 3.4166667W)	No match
				***psb*A**		
SSH4+5 (91)	JQ422235	99.8%	*Lithothamnion glaciale*	GWS007542	N/A	N/A
SSH4+5 (1444)	JQ422235	99.7%	*Lithothamnion glaciale*	GWS007542	N/A	N/A
SSH12 (47)	GQ917708	100%	Uncultured Corallinales	LBC0013	France (48.731937N 3.939152W)	N/A

*Voucher LBC0001 has been subsequntly assigned to *Phymatolithon* sp. by Bittner *et al.* (2011).

CHAPTER 2

Phymatolithon lusitanicum sp. nov. (Hapalidiales, Rhodophyta): the third most abundant maerl-forming species in the Atlantic Iberian Peninsula

This chapter is based on content published in:

Peña V., Pardo C., López L., Carro B., Hernández-Kantún J., Adey W. H., Bárbara I., Barreiro R. & Le Gall L. (2015). Phymatolithon lusitanicum sp. nov. (Hapalidiales, Rhodophyta): the third most abundant maerl-forming species in the Atlantic Iberian Peninsula. Cryptogamie, Algologie 36, 429-459.

PHYMATOLITHON LUSITANICUM SP. NOV. (HAPALIDIALES, RHODOPHYTA): THE THIRD MOST ABUNDANT MAERL-FORMING SPECIES IN THE ATLANTIC IBERIAN PENINSULA

ABSTRACT

Phymatolithon lusitanicum is a new maerl species described based on an integrative systematic approach including molecular (COI-5P, *psb*A) and morphological data obtained from recent collections, as well as comparison of type material from the morphologically and ecologically alike NE Atlantic species *P. lamii* and *P. laevigatum*. Molecular analyses including type material of *P. lamii* and *P. laevigatum* were congruent in delimiting *P. lusitanicum* as an independent lineage from these crustose species. The three species shared a common external morphology of multiporate asexual conceptacles, but *P. lusitanicum* has been detected only unattached as maerl while *P. lamii* and *P. laevigatum* are crustose. *Phymatolithon lusitanicum* is particularly abundant in subtidal maerl beds of the Atlantic Iberian Peninsula (Galicia and the Algarve); however it has also been detected northwards in Ireland intertidally and in Western Mediterranean Sea (Alborán Sea, Balearic Islands) down to 64 m. *Phymatolithon lusitanicum* differs from other *Phymatolithon* species reported for the European coasts mainly by the external shape of the multiporate asexual conceptacles (pore plate flush with surface or slightly sunken without a conspicuous thick raised rim) and its unattached habit as maerl/rhodolith. In addition, the lectotype of *Lithothamnion hamelii* turned out to be conspecific to *Phymatolithon calcareum*, therefore this taxon is proposed as a heterotypic synonym of *P. calcareum.* Finally, our molecular analyses detected cryptic diversity within the European collections of *Phymatolithon*, while collections identified as *P. lenormandii* from Canada or *P. repandum* from New Zealand were resolved as unrelated to the remaining *Phymatolithon.* In the light of these results, it is clear that further work is necessary to resolve species diversity within the genus *Phymatolithon* and its relationship with related genera.

KEYWORDS: Biodiversity, DNA barcoding, *Lithothamnion hamelii,* Mediterranean, NE Atlantic, *Phymatolithon laevigatum, Phymatolithon lamii,* systematics.

INTRODUCTION

The genus *Phymatolithon* Foslie contains non-geniculate coralline algae that include 14 currently accepted names for which seven species are reported in Europe (Guiry & Guiry 2015) associated to coastal habitats from the intertidal to the low subtidal (Adey & Adey 1973, Chamberlain & Irvine 1994, Teichert *et al.* 2012). *Phymatolithon* together with *Lithothamnion* Heydrich have been regarded as the major contributors to European maerl beds in terms of species diversity as well as abundance and distribution range (Chamberlain & Irvine 1994, Babbini & Bressan 1997, Bressan & Babbini 2003). In particular, *Phymatolithon calcareum* is commonly cited in the literature as the main builder of the Atlantic and Mediterranean maerl beds (Chamberlain & Irvine 1994, Bressan & Babbini 2003). *Phymatolithon purpureum* has also been reported as a maerl-forming species in Ireland, and more recently in the Mediterranean (as *P. polymorphum* (Linnaeus) Foslie in Adey & Adey 1973, Blunden *et al.* 1981, Hernández-Kantún *et al.* 2014, 2015b), but it is more commonly found as an Atlantic encrusting species, epilithic or epiphytic on kelp holdfasts and stipes, from the low littoral to deep subtidal down to 45 m. A similar habit is reported for *Phymatolithon laevigatum, P. lamii* and *P. lenormandii* which commonly occur in the Atlantic coasts as crustose attached plants from the mid-lower littoral to 30-35 m depth (down to 90 m for *P. lamii*, Adey & Adey 1973, Chamberlain & Irvine 1994). The latter two species (*P. lamii* and *P. lenormandii*) have been also cited in the Mediterranean as encrusting species, epilithic or epizoic on *Chlamys* sp. shells, down to 55 m depth (Bressan & Babbini 2003, Kaleb *et al.* 2012). By contrast, *Phymatolithon brunneum* has been described as a rare, epilithic plant that occurs in the littoral above the *P. purpureum* zone or even higher up the shore, but only in Britain, Channel Islands and Atlantic France (Chamberlain & Irvine 1994). Other rare species is *P. tenuissimum* (Foslie) Adey which has been cited in the Mediterranean, and formerly described in São Tomé, West coast of Africa (Foslie 1900, Babbini & Bressan 1997). Apart from the growth-form and habitat, the external morphology of multiporate sporangial conceptacles has been usually employed in the literature for the identification of European *Phymatolithon* taxa (Chamberlain & Irvine 1994, Bressan & Babbini 2003).

In the Atlantic Iberian Peninsula, other than the maerl-forming *P. calcareum* (Carro *et al.* 2014, Pardo *et al.* 2014a, Peña *et al.* 2014b), the only members of the genus reported so far are *P. lenormandii, P. lamii* and *P. purpureum*, but always as attached crustose plants (Ardré 1970, Adey & Adey 1973, Chamberlain 1991, Chamberlain & Irvine 1994, Bárbara *et al.* 2003). Nonetheless, new research with DNA barcodes recently detected a still undescribed species in

Iberian maerl beds that, according to sequence information derived from mitochondrial and plastid markers (COI-5P and *psb*A), was tentatively assigned to *Phymatolithon* sp.3. Interestingly, this new species was a common component of maerl beds in Galicia, where it appeared mixed with the widespread *P. calcareum* and *Lithothamnion corallioides*, and it even became the dominant component of maerl beds southwards in the Algarve (Carro *et al.* 2014, Pardo *et al.* 2014a). Additional morphological studies provided further support to the molecular-based delimitation, while the observation of multiporate asexual conceptacles with pore plates lightly protruding or flush with the surface suggested some similarities with *P. lamii* or *P. laevigatum* (Chamberlain 1991, Chamberlain & Irvine 1994). Nevertheless, no name could be assigned with certainty given the absence of molecular data from any other type species reported in European coasts other than the generitype *P. calcareum* (Pardo *et al.* 2014a, Peña *et al.* 2014b, Hernández-Kantún *et al.* 2015b). Alternatively, topotype material (specimens of the targeted species from the type locality) could be studied, but it should be done with caution given the frequent co-ocurrence of several *Phymatolithon* taxa in the same habitat. Here, we formally describe the maerl species *Phymatolithon lusitanicum* sp. nov. on the basis of novel molecular data obtained from type material of *P. lamii* and *P. laevigatum* and from recent collections of other European *Phymatolithon* species.

MATERIAL AND METHODS

Collections studied

Maerl specimens identified as *Phymatolithon* sp.3 using DNA barcoding (COI-5P/*psb*A) have been collected in recent surveys of maerl beds in the Atlantic Iberian Peninsula both in Galicia (Spain) and Algarve (Portugal) (Carro *et al.* 2014, Pardo *et al.* 2014a). Intertidal samples identified in the field as *Phymatolithon* spp. were collected on rocky shores using a hammer and chisel in several sites along the Atlantic European coasts (France, Spain and Portugal) including the type locality of *P. purpureum* (Fort du Mingant, Brittany, France, Chamberlain & Irvine 1994). In addition, intertidal and subtidal collections from Ireland and Western Mediterranean Sea (Alborán Sea) have been also studied (**Table S1**). Samples were air-dried or oven-dried (50º C), preserved in zipper bags with silica gel before molecular and morphological studies, and deposited in the herbaria of Universidade de Santiago de Compostela (SANT), Museum National d´Histoire Naturelle (PC), Smithsonian Institution (US) and National University of Ireland (GALW) (acronyms follow Thiers 2015). In addition, we studied the type material of *Lithophyllum lamii* Me. Lemoine (lectotype, PC0719024), *Lithothamnion laevigatum*

Foslie (paratype, PC0145173), *Lithothamnion purpureum* P.L.Crouan & H.M.Crouan (lectotype-CO02256, and isotype-CO02260), *Melobesia lenormandii* Areschoug (isolectotype, CN) and *Lithothamnion hamelii* (lectotype, PC0145174) deposited in the herbaria of Concarneau (CO), the Université de Caen (CN) and the Museum National d´Histoire Naturelle (PC) (**Table S1**, Chamberlain 1991, Chamberlain & Irvine 1994, Woelkerling & Lamy 1998).

Molecular studies

A total of 38 specimens from recent intertidal and subtidal collections were cleaned under a stereomicroscope and selected surfaces were ground for DNA extraction. Genomic DNA was extracted using a NucleoSpin® 96 Tissue kit (Macherey-Nagel, GmbH and Co. KG, Germany), or the QIAamp® DNA Micro Kit (Qiagen S.A.S., France, protocol for tissues) for the type material. Samples from Ireland (E302, E113, E17, E10, **Table S1**) were extracted following Hernández-Kantún *et al.* (2014), while US herbarium vouchers identified as *P. rugulosum* and *P. lenormandii* followed Adey *et al.* (2015). The mitochondrial COI-5P fragment was PCR-amplified using primer pairs GazF1/GazR1, GazF1/GCorR3, or GWSFn/GWSRx (Saunders 2005, Le Gall & Saunders 2010b, Saunders & McDevit 2012b, Pardo *et al.* 2014a, Peña *et al.* 2015a). The *psb*A locus was amplified using primer pairs *psb*A-F1/*psb*A-R2 or *psb*A-F1/*psb*A600R (Yoon *et al.* 2002). Thermal profiles for PCR amplification of COI-5P and *psb*A fragments followed Saunders & McDevit (2012b), and Bittner (2009), respectively. PCR reactions followed Peña *et al.* (2015a). DNA extractions and amplifications of type material were performed separate from recent collections, and with negative controls run throughout. PCR products were purified and sequenced by Genoscope (Bibliothèque du Vivant program, Centre National de Séquençage, France); except for those obtained from specimens from Ireland or US specimens that followed Hernández-Kantún *et al.* (2014), and Adey *et al.* (2015), respectively. Sequences were assembled and aligned with the assistance of CodonCode Aligner® (CodonCode Corporation, USA) and adjusted by eye using SeaView v.4 (Gouy *et al.* 2010). Sequences were submitted to the Barcode of Life Data Systems (project "NGCOR", BOLD, http://www.boldsystems.org; Ratnasingham & Hebert 2007), and GenBank (accession numbers listed in **Table S1**). Our analyses also included COI-5P and *psb*A sequences of *Phymatolithon* sp.3 generated in previous studies of Atlantic Iberian maerl beds (Carro *et al.* 2014, Pardo *et al.* 2014a), as well as publicly available sequences assigned to the genus *Phymatolithon* in BOLD and GenBank databases, of the latter included other maerl-forming species detected in the OSPAR region (BOLD project "MAERL", Pardo *et al.* 2014a). Finally, the alignments comprised 177 COI-5P and 42 *psb*A sequences, including the generitype *P.*

calcareum (Pardo *et al.* 2014a, Peña *et al.* 2014b, Hernández-Kantún *et al.* 2015b) and other European taxas in the subfamily Melobesioideae (*Lithothamnion* and *Mesophyllum*) that were used as outgroups (**Table S1**). A General Mixed Yule Coalescent (GMYC) model was applied to delimit *Phymatolithon* species (Pons *et al.* 2006, Fujisawa & Barraclough 2013). The ultrametric tree derived from Bayesian phylogenetic analyses of the COI-5P alignment was ran in BEAST v.1.7.4 (Drummond *et al.* 2012) under a Generalized Time-Reversible model with Gamma distribution plus Invariant sites (GTR+G+I) to accommodate rate heterogeneity, an UnCorrelated Log Normal (UCLN) relaxed molecular clock, and using a coalescence tree prior. In BEAST, two Markov Chain Monte Carlo (MCMC) analyses were run for 10 million generations, sampling every 1000th generation. The information from a simple of trees was summarized onto a single "target" tree (10% burn-in discarded at the start of the run, 0.5 of posterior probability limit of the nodes in target tree) using Tree Annotator v.1.7.4 (http://beast.bio.ed.ac.uk). GMYC analyses were performed using the SPLITS package for R (http://r-forge.r-project.org/project/splits). Phylogenetic relationships were inferred using Maximum Likelihood (ML) and Bayesian Inference (BI) using Mega v.6.0 (Tamura *et al.* 2013) and MrBayes v.3.2.1 (Ronquist & Huelsenbeck 2003), respectively. Models of sequence evolution were estimated using the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC) obtained in jModeltest v.2.1.3 (Darriba *et al.* 2012). Maximum likelihood and Bayesian analyses for the COI-5P and *psb*A alignments were performed under a GTR+G+I model. The Bayesian analyses were performed under the same model with four MCMC for 10 million generations, and tree sampling every 1000 generations.

Morphological studies

Representative specimens of *Phymatolithon lusitanicum* and other *Phymatolithon* species from the Atlantic Iberian Peninsula were selected for anatomical examination with a Scanning Electron Microscope (SEM, model JEOL JSM 6400, Universidade da Coruña, Spain). SEM analyses for specimens from Ireland followed Hernández-Kantún *et al.* (2014). Particular attention was directed towards key features historically used to delimit *Phymatolithon* species regarding habit and growth form, and characters related to the multiporate sporangial conceptacles such as external shape and the presence or absence of a conspicuous rim (Chamberlain 1991, Chamberlain & Irvine 1994, Kaleb *et al.* 2012). The anatomical terms medulla (equivalent to the terms hypothallium and core) and cortex (equivalent perithallium and peripheral region) follows (Chamberlain & Irvine 1994). Cell length is the distance between primary pit-connections, and cell diameter is the measurement taken perpendicularly to this

across the middle of the cell lumen. Conceptacle measurements were taken according to Adey & Adey (1973) and Chamberlain & Irvine (1994).

RESULTS

The COI-5P alignment of 177 sequences, including publicly available sequences from GenBank and BOLD, ranged from 538 to 664 base pairs (bp), and consisted of 46 haplotype sequences with 262 variable sites. The phylogenetic tree obtained from the ML analysis of the COI-5P alignment resolved *Phymatolithon* taxa studied into 12 supported lineages, 10 of them corresponding to European collections (**Fig. 1**, **Table S1**). Infralineage variation (uncorrected *p*-distance) ranged from 0% to 1.8%. According to the GMYC model, the COI-5P alignment supported the 12 *Phymatolithon* lineages (**Fig. 1**), and it estimated a total of 17 putative Hapalidiales species with a confidence interval ranging from 16 to 19 taxa. The fit of the likelihood of the GMYC model was significantly higher ($P < 0.001$) than that of a null model of uniform coalescent branching rates. The threshold time (estimated depth from the branch tips at which the transition from population to species level branching patterns occur) was determined at -0.00686 substitutions per site. Both GYMC and ML analyses of COI-5P data were congruent in delimiting the maerl collections from Atlantic Iberia and western Mediterranean Sea as an independent lineage separated from crustose specimens identified as *P. lamii*. Based on our collections, the diversity of *Phymatolithon* taxa in the European coasts is high (10 putative species, **Fig. 1**). It is noteworthy that the two Canadian collections identified as *P. lenormandii* were remotely related to the remaining *Phymatolithon* included in the analyses (**Fig. 1**).

The *psb*A alignment comprised 42 sequences ranging from 543 to 851 bp, with 204 variable sites. It included sequences from the type material of *Phymatolithon calcareum, P. lamii, P. laevigatum* and *Lithothamnion hamelii* while our attempts to amplify the *psb*A region for the type material of *P. lenormandii* and *P. purpureum* were unsuccessful (**Fig. 2**). According to the molecular information obtained from type material of *Phymatolithon lamii*, our crustose collections were correctly identified (match = 98.9-99.1 %). Among the specimens identified as *P. lamii* were those collected at the type locality of *P. purpureum* (Fort du Mingant, France, VPF00223 as KT807942 represented in **Fig. 2**, together with VPF00223 as KT807914, VPF00225

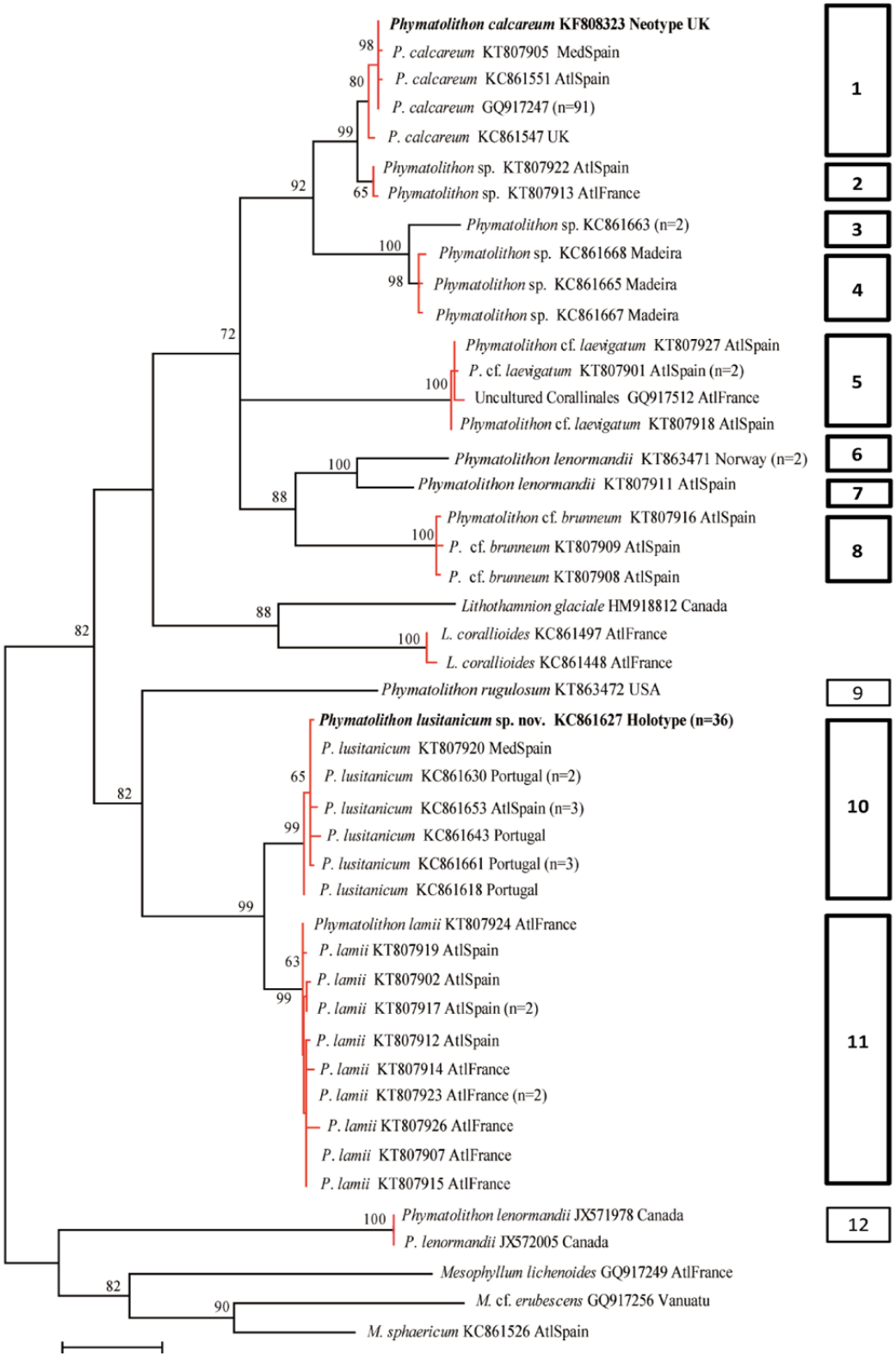


Fig. 1. ML tree inferred from DNA barcode (COI-5P) for each species delimited according to the GMYC model (red colour). In bold, holotype of *Phymatolithon lusitanicum* sp. nov. and neotype of *P. calcareum*. The twelve supported lineages of *Phymatolithon* are indicated; in bold, the European ones. Bootstrap values > 60 % are shown for each node. Scale bar: 0.05 substitutions per site.

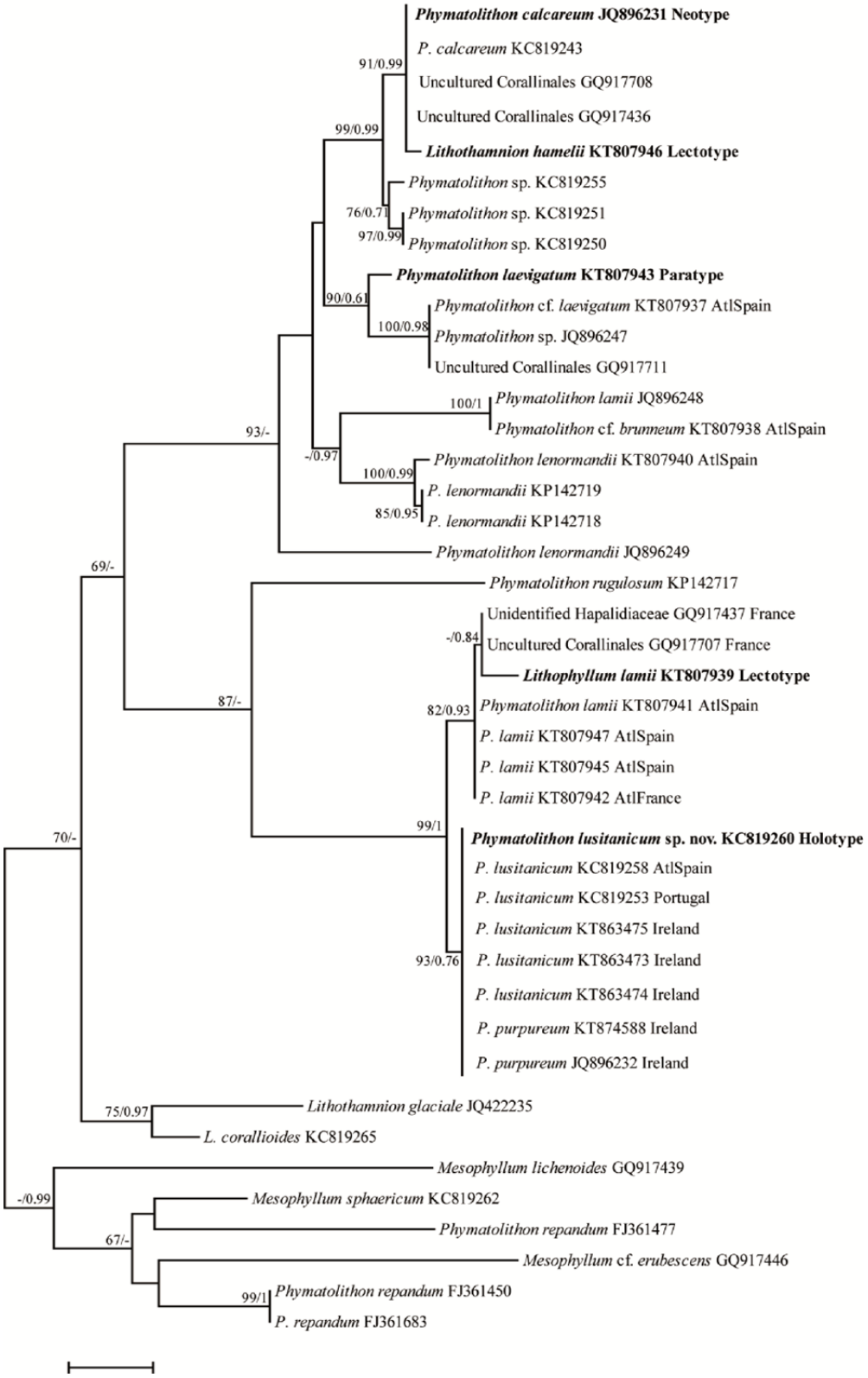


Fig. 2. Phylogenetic tree inferred from ML and BI analyses of *psb*A sequences of European *Phymatolithon* and publicly available sequences for this genus. In bold, holotype of *Phymatolithon lusitanicum* sp. nov., and type material of *P. calcareum, P. lamii, P. laevigatum* and *Lithothamnion hamelii*. Bootstrap ML values > 60% and posterior probabilities > 0.60 from Bayesian inference are shown for each node. Members of subfamily Melobesioideae were used as outgroup. Scale bar: 0.02 substitutions per site.

as KT807907, and VPF00236 as KT807926, represented in **Fig. 1**, **Table S1**), indicating that the specimens belong to *P. lamii* and not to *P. purpureum*. Both ML and BI resolved the maerl-forming *P. lusitanicum* as a sister taxon to *P. lamii* with full support (99%/1 for ML and BI, respectively; **Fig. 2**). The clade of *P. lusitanicum* comprised collections from the Iberian Peninsula as well as maerl specimens from Ireland, the latter included one specimen previously assigned to *P. purpureum* (E28 as JQ896232, E10 as KT863474, E17 as KT863473, E113 as KT874588, and E302 as KT863475; **Fig. 2**, **Table S1**). Additionally, the paratype of *P. laevigatum* was closely related to collections from Ireland and from the Atlantic coasts of France and Spain, while the lectotype of *Lithothamnion hamelii* was conspecific with the neotype (Falmouth, England) and recent collections of *Phymatolithon calcareum* from Atlantic Europe (**Fig. 2**, **Table S1**). On the other hand, specimens identified as *P. lenormandii* from Norway, Ireland and Atlantic Spain represented three different clades within the genus *Phymatolithon* (**Fig. 2**). By contrast, New Zealand collections of *P. repandum* were resolved as unrelated with other *Phymatolithon* species included in our analyses.

Given the molecular evidence shown above, we proposed to describe the new species of maerl as *Phymatolithon lusitanicum.*

Phymatolithon lusitanicum V. Peña sp. nov. (**Fig. 3-5**)

Diagnosis: plant unattached, monomerous, scarcely to densely branched (**Fig. 3)**. Epithallial cells disposed in 1 (2) layers, cells domed, 1.5-2.5 µm long by 4 µm wide in vertical section (**Fig. 4c),** 5-6 µm in diameter in surface view, polygonal with thick walls (**Fig. 4d**). Subepithallial initials as short as or shorter than cells subtending them, 2-3 µm long by 4-8 µm wide. Cortex composed of cells 10-14 µm long by 5-7 µm wide. Cell fusions present (**Fig. 4c**). Secondary pitconnections absent. Trichocytes absent. Sporangial conceptacles multiporate with a white color, without a conspicuous, thick raised rim, sometimes covered by a calcified cap (**Fig. 3i, Fig. 5a,d**), pore plate flushed with surface or slightly sunken, up to 30 pores, 130-170 µm in diameter (**Fig. 5b,c**). Chambers elliptical, (35) 40-75 µm high by 70-166 (175) µm wide with roof composed of 2-3 (4) cells, 12-25 µm thick (**Fig. 5d-f**). Buried conceptacles within the tissue not observed. Sexual uniporate conceptacles unknown.

Morphology: Non-geniculate, unattached, sparsely to densely branched (up to fifth-order branching), size ranged 0.20–7.88 cm^3, shape mainly discoidal and ellipsoidal (**Fig. 3a-i**). Colour pink -greyish to pale pink- to purple, texture smooth and matt when dried.

Anatomy: Pseudoparenchymatous, monomerous, and radially organized (**Fig. 4a,b**). Medullary cells 4–7 µm in diameter, cortical cells 10–14 µm x 5–7 µm. The subepithallial initials are as short as or shorter than cells subtending them, 2–3 µm long by 4–8 µm in diameter (**Fig. 4c**). Each cortical filament produces one epithallial cell domed in transverse section; they are 1.5–2.5 µm long by 4 µm wide, and are disposed in 1 (2) layers (**Fig. 4c**); in surface view (**Fig. 4d**), epithallial cells are 5–6 µm in diameter, polygonal with thick walls of 2–3 µm (*Phymatolithon*-type SEM, as suggested by Chamberlain & Irvine 1994). Fusions cells present between cells of neighbouring filaments, secondary pit connections are absent (**Fig. 4c**). Trichocytes are absent.

Reproductive structures: Sexual uniporate conceptacles not observed. Sporangial conceptacles multiporate, white coloured, without a conspicuous, thick raised rim, sometimes covered by a calcified cap (**Fig 3i** and **Fig. 5a,d**), pore plate flush with surface or slightly sunken, 130–170 µm in diameter, up to 30 pores (**Fig. 5b,c**). Chambers were found empty, elliptical, (35) 40–75 µm high by 70–166 (175) µm wide, roof of mature conceptacles composed of 2–3 (4) cells, 12–25 µm thick (**Fig. 5d,e,f**). Tetrasporangia or bisporangia not observed. Buried multiporate senescent conceptacles not observed within the thallus. Sporangial conceptacles

occasionally recorded, from the Algarve collections in May, June and September, and from Ireland in autumn (September- November).

Holotype: voucher CPVP-676 (COI-5P: KC861627; *psb*A: KC819260), Con de Pego, Ría de Vigo, Galicia, Spain; 42º 15.498 N, 8º 45.087 W, 5 m depth, collectors V. Peña, I. Bárbara & R. Barreiro, 05/04/2011, SANT-Algae 29522 (**Fig. 3d**, **Table S1**). Isotypes: CPVP-689 (COI-5P: KC861620; SANT-Algae 29523) and CPVP-685 (COI-5P: KC861621; SANT-Algae 29524).

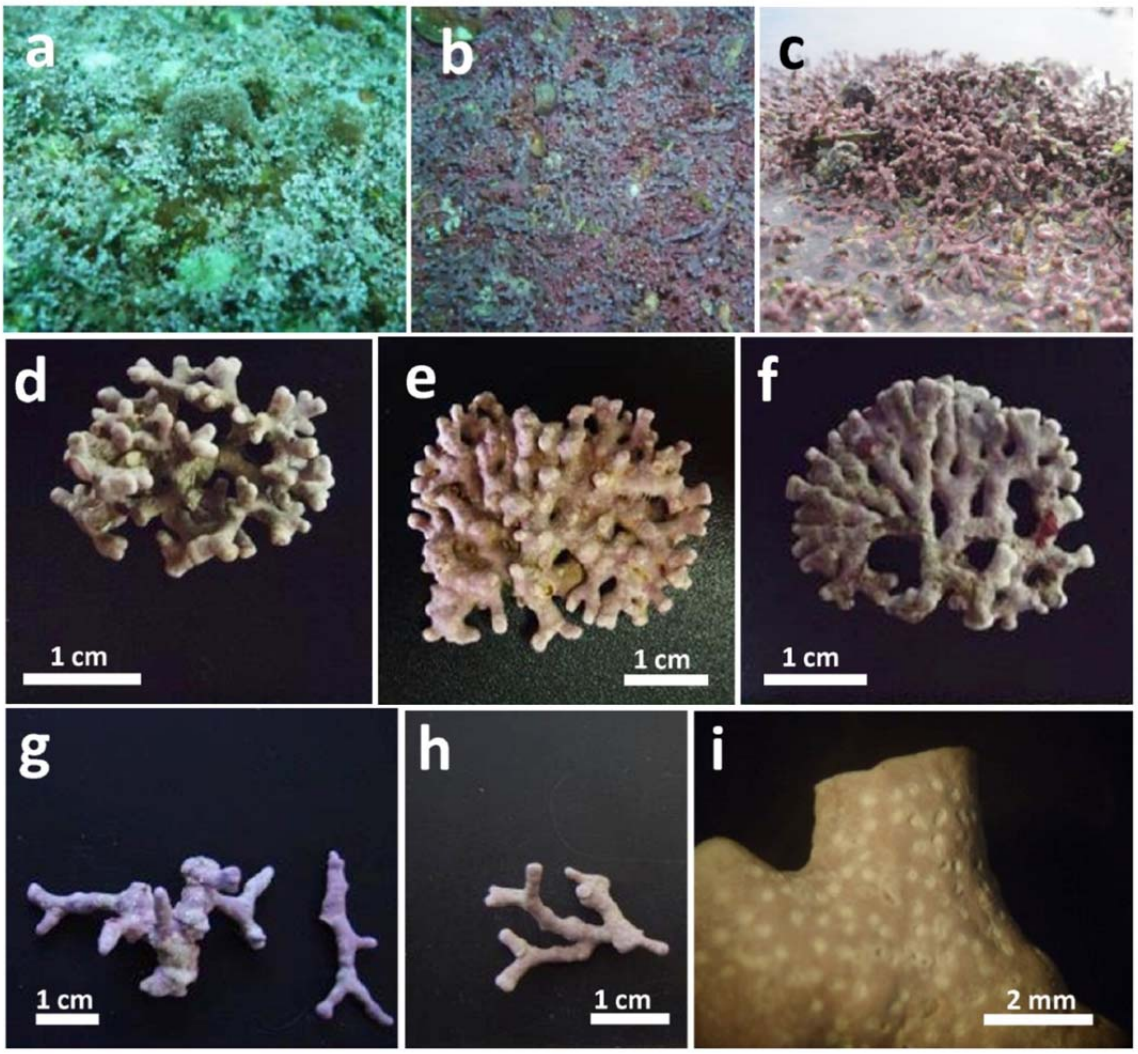
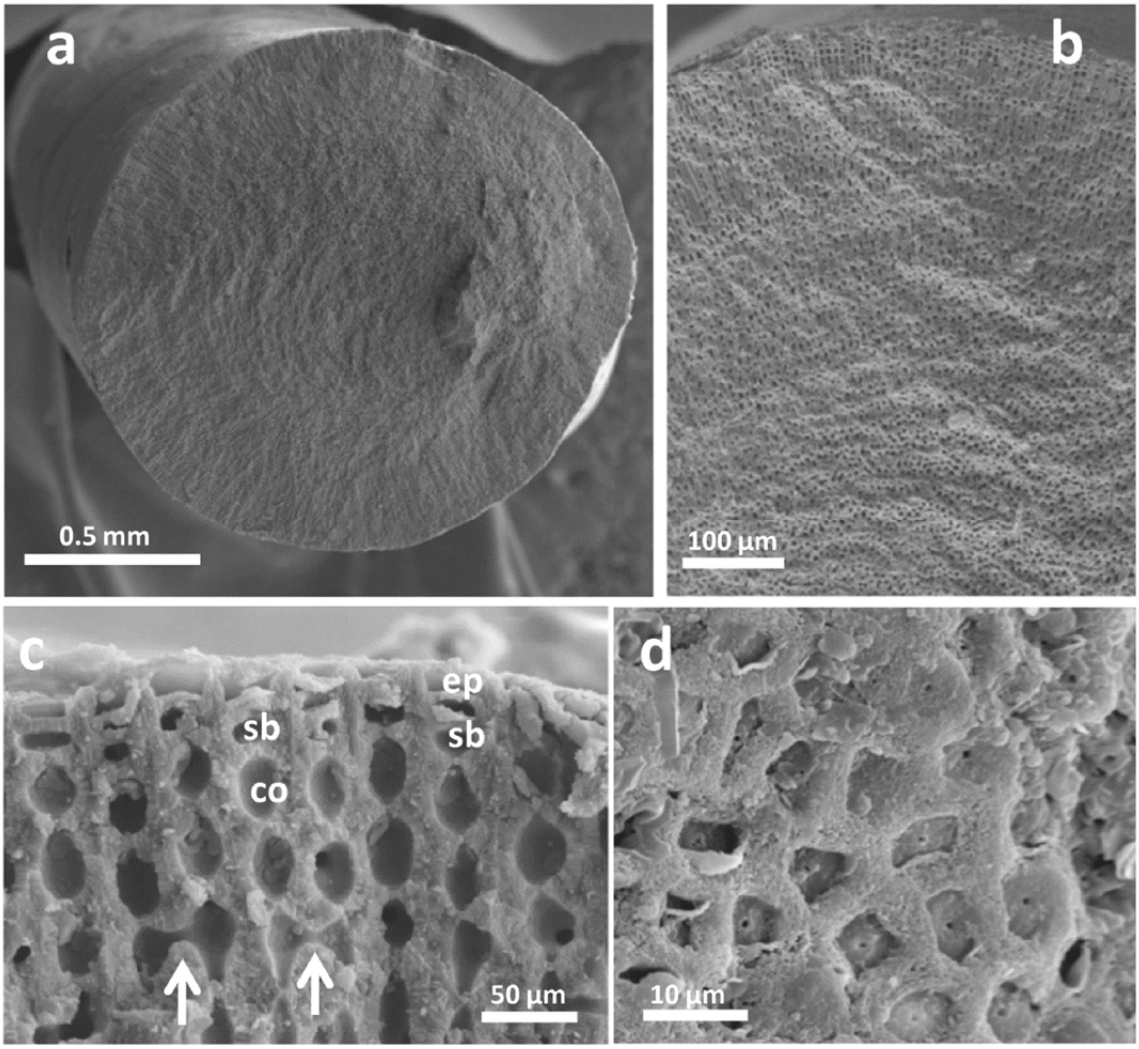


Fig. 3. *Phymatolithon lusitanicum* sp. nov. **a.** *Phymatolithon lusitanicum* mixed with *P. calcareum* and *Lithothamnion corallioides* at the type locality (Con de Pego, Ría de Vigo, Galicia). **b.** *Phymatolithon lusitanicum* forming maerl at 20 m depth (Armaçao de Pêra, Algarve, south Portugal). **c.** Intertidal maerl of *P. lusitanicum* in Muckinish, Ireland. **d.** Holotype of *P. lusitanicum* showing an ellipsoidal shape (voucher SANT-Algae 29522). **e-f**. Discoidal specimens of *P. lusitanicum* collected in Galicia and the Algarve (vouchers SANT-Algae 29547 and SANT-Algae 29517, respectively). **g-h.** Collections of *P. lusitanicum* in Alborán Sea, Mediterranean (vouchers SANT-Algae 29506 and SANT-Algae 29508, respectively). **i.** Maerl branch showing multiporate asexual conceptacles without conspicuous raised rim and white pore plate (Armaçao de Pêra, Algarve, south Portugal, SANT-Algae 29509).



F**ig. 4**. *Phymatolithon lusitanicum* sp. nov. **a-b.** Vertical section of the branch showing monomerous structure (voucher SANT-Algae 29534). **c.** Vertical section showing domed epithallial cells (ep), subepithallial cells (sb) as short as or shorter than cells subtending them, and cortical cells (co) joined by cell fusions (arrows) (voucher SANT-Algae 29534). **d.** Surface view of epithallial cells polygonal with thick walls (*Phymatolithon*-type, voucher SANT-Algae 29510)

Complementary collections: voucher CPVP-639 (COI-5P: KC861642, *psb*A: KC819258), Playa de Tulla, Ría de Pontevedra, Galicia, Spain, 9 m depth, collectors V. Peña, I. Bárbara & R. Barreiro, 07/04/2011, SANT-Algae 29547 (**Fig. 3e**); voucher CPVP-478 (COI-5P: KC861630; *psb*A: KC819253), Armaçao de Pêra, Algarve, Portugal, 20 m depth; collectors P. Neves & M. Rodrigues, 02/03/2011, SANT-Algae 29517 (**Fig. 3f**); voucher VPF00451 (COI-5P KT807910), Alborán Sea, Mediterranean Sea, 48 m depth, collector INDEMARES project, 24/04/2011, SANT-Algae 29506 (**Fig. 3g**); voucher VPF00511 (COI-5P KT807910), Alborán Sea, Mediterranean Sea, 40–44 m depth, collector INDEMARES project, 22/04/2011, SANT-Algae 29508 (**Fig. 3h**); voucher SANT-Algae 29509, Armaçao de Pêra, Algarve, Portugal, 20 m depth;

collectors V. Peña & I. Bárbara, 02/04/2008, (**Fig. 3i**); voucher SANT-Algae 29510, Armaçao de Pêra, Algarve, Portugal, 20 m depth; collectors V. Peña & I. Bárbara.

Etymology: The specific epithet refers to the range of distribution in which the species is more abundant, the Lusitania biogeographical province.

Habitat: *Phymatolithon lusitanicum* mainly occurs subtidally as maerl (rhodoliths) in relatively shallow beds in Galicia (4–13 m, **Fig. 3a**), and in Ireland (6 m), but deeper in southern Portugal (15–20 m, **Fig. 3b**), in the Mediterranean Alborán Sea (40–48 m), and in the Balearic Islands (54–64 m, Hernández-Kantún *et al.* 2014, as *P. purpureum*). The species is uncommonly reported from an intertidal bed in Muckinish, Ireland (E302, **Table S1**, **Fig. 3c**, Hernández-Kantún *et al.* 2014, as *P. purpureum*). In Galicia, *P. lusitanicum* is usually found mixed with *P. calcareum* and *Lithothamnion corallioides* (Carro *et al.* 2014, Pardo *et al.* 2014a), and with *Mesophyllum sphaericum* (Peña *et al.* 2011) in the only locality where the latter has been reported in the Atlantic Iberian Peninsula.

Distribution: Ireland (Kingstown Bay, Carraroe beach and Muckinish), Atlantic Iberian Peninsula (Galicia and the Algarve), and western Mediterranean Sea (Alborán Sea and the Balearic Islands).

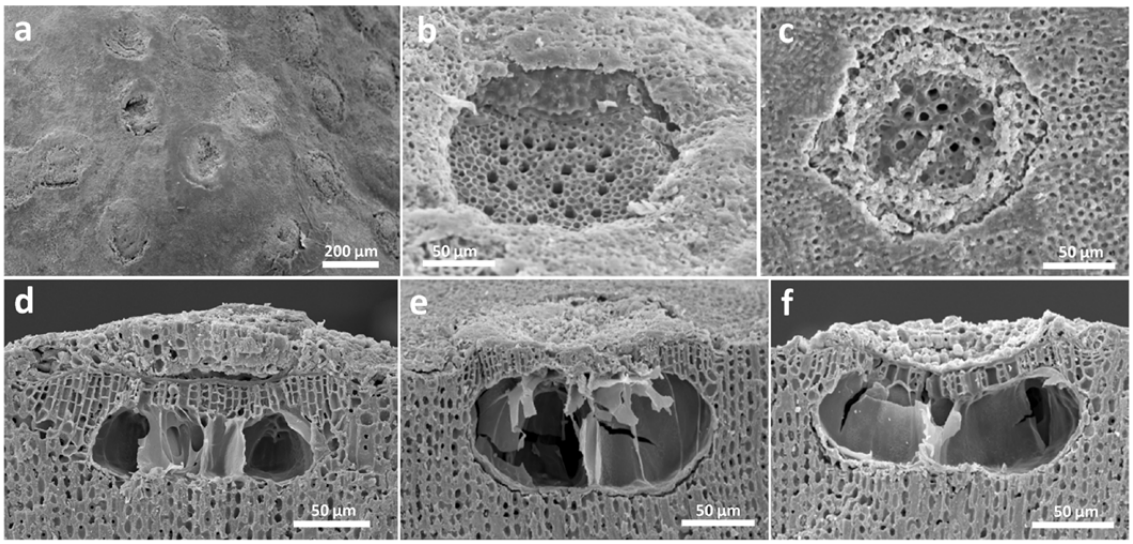


Fig. 5. *Phymatolithon lusitanicum* sp. nov. a. Surface view of multiporate asexual conceptacles with calcified cap, without a conspicuous, thick raised rim (voucher E302, SANT-Algae 29597). b-c. Surface view of multiporate asexual conceptacles without a conspicuous raised rim and pore plate flush with the surface or slightly sunken (voucher SANT-Algae 29509). d. Vertical section of a multiporate sporangial conceptacle covered by a calcified cap (voucher E302, SANT-Algae 29597). e-f. Vertical section showing multiporate sporangial conceptacles with an elliptical chamber empty and clearly laminated, roof composed of 2-3 (-4) cells thick (voucher E302, SANT-Algae 29597).

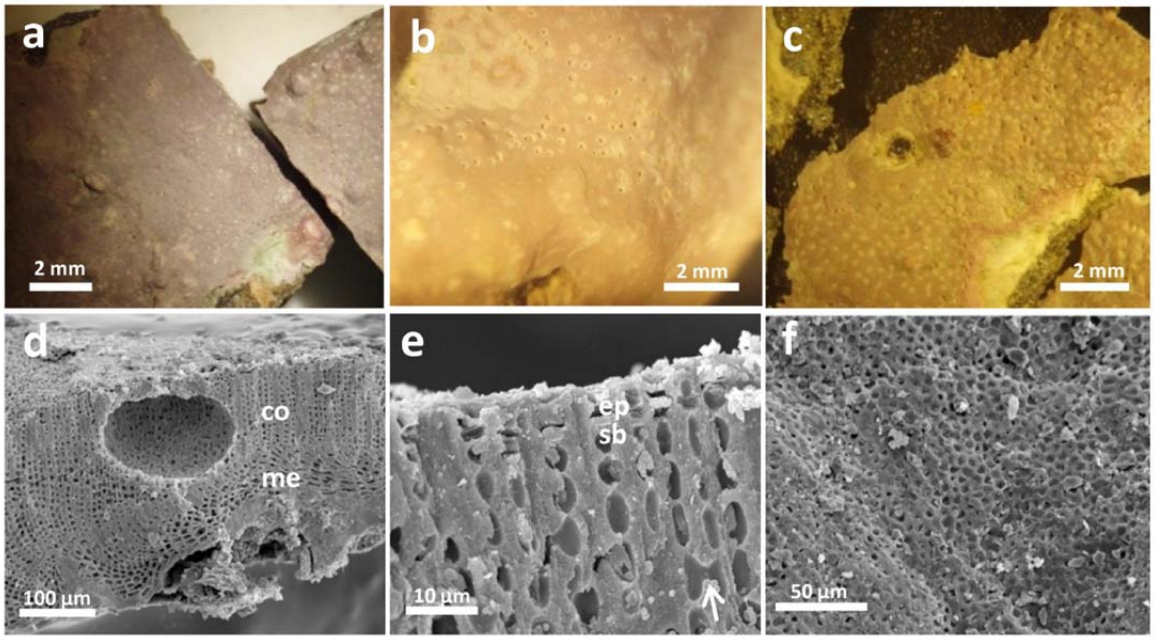


Fig. 6. *Phymatolithon lamii.* **a-c.** Crustose, epilithic specimens showing conceptacles without conspicous raised rim, pore plates white-coloured, initially sunken forming deep holes or flush with surface at maturity (vouchers SANT-Algae 29501, SANT-Algae 29477 and SANT-Algae 29491, respectively). **d.** Vertical section showing medullary (me) and cortical cells (co), and uniporate sexual conceptacle empty (voucher SANT-Algae 29501). **26.** Vertical section showing domed epithallial cells (ep), subepithallial cells (sb) as short as or shorter than cells subtending them, and cortical cells joined by cell fusions (arrow) (voucher SANT-Algae 29498). **27.** Surface view of epithallial cells polygonal with thick walls (*Phymatolithon*-type, voucher SANT-Algae 29498).

Comments: Phymatolithon lusitanicum differs from other *Phymatolithon* species reported from European coasts mainly by the external shape of the multiporate asexual conceptacles (pore plate flush with surface or slightly sunken and without a conspicuous, thick raised rim), and its unattached habit as maerl/rhodolith. In addition, recent collections of *P. lamii* matched the habit and ecology described in the literature (Chamberlain 1991, Chamberlain & Irvine 1994, Kaleb *et al.* 2012) because they were crustose, epilithic specimens with uniporate sexual and multiporate asexual conceptacles appearing initially as deep holes, or more or less flush with surface and showing white pore plates at maturity (**Fig. 6a-f**). They were collected from the intertidal to the shallow subtidal (down to 10 m), in rocky shores but also associated with maerl beds in Brittany (**Table S1**).

DISCUSSION

Phymatolithon lusitanicum is described as the third major maerl-forming species in the Atlantic Iberian Peninsula although its distribution range extends to subtidal and also intertidal maerl beds from Ireland to Western Mediterranean Sea (Alborán Sea and the Balearic Islands). In Galician maerl beds, *P. lusitanicum* has been usually detected mixed with *P. calcareum* and

Lithothamnion corallioides as well as *Mesophyllum sphaericum*, although in southern Portugal it is reported as the dominant maerl species (Carro *et al.* 2014, Pardo *et al.* 2014a, as "*Phymatolithon* sp.3"). Based on this evidence, it is likely that some of the previous records of *P. calcareum* in the Atlantic Iberian Peninsula (Peña & Bárbara 2008a, Hall-Spencer *et al.* 2010), and particularly in southern Portugal (Peña *et al.* 2009), might be actually referred to *P. lusitanicum*. Likewise, the detection of *P. lusitanicum* in maerl collections from Ireland that had previously been assigned to *P. purpureum* (E28, E113, Hernández-Kantún *et al.* 2014, Hernández-Kantún *et al.* 2015b) suggests that other misidentifications may exist in the literature (Adey & Adey 1973, Blunden *et al.* 1981), and highlights that more detailed studies are required to get a better delimitation of these two species and to clarify the occurrence of *P. purpureum* as a maerl-forming species. According to Chamberlain & Irvine (1994), *P. purpureum* is characterized by showing multiporate sporangial conceptacles with thick, tyre-like, raised rims up to 100 μm wide. Similarly, the Mediterranean maerl collections from the Balearic Islands originally assigned to *P. purpureum* (E242, E245, E269-E270 in Hernández-Kantún *et al.* 2014) must be considered *P. lusitanicum* according to their conspecificity with Irish collections (E113), challenging the occurrence of *P. purpureum* in the Mediterranean. According to the evidence presented here and in previous studies that used molecular tools to investigate extensive collections of maerl along Atlantic European coasts (Carro *et al.* 2014, Pardo *et al.* 2014a, Hernández-Kantún *et al.* 2015b), *P. lusitanicum* reaches western Ireland in the north but has gone undetected in Britain and France. In this regard, the disjunct distribution of *P. lusitanicum* contrasts with the pattern shown by the other two maerl species that typically occur mixed with it and that overwhelmingly dominate maerl beds in France and southern Britain (*P. calcareum* and *Lithothamnion corallioides*). Hence, further research with appropriate molecular tools on maerl beds from both regions (Britain, France) are required to clarify the discontinuity observed in the range of *P. lusitanicum*.

The collections studied here together with results derived from Hernández-Kantún *et al.* (2014, 2015b) show that *Phymatolithon lusitanicum* occurs in shallow maerl beds in Galicia and in Ireland (even intertidally), while it was found at greater depths (from 15-64 m) in Algarve and Western Mediterranean Sea (Alborán Sea, Balearic Islands). The migration to greater depths from the Atlantic to the Mediterranean is shared with other Atlantic maerl-forming taxa recorded in the Mediterranean (*P. calcareum, Lithothamnion corallioides, Mesophyllum sphaericum*) as well as with other non-geniculate species (*M. expansum* (Philippi) Cabioch & M.L. Mendoza) (Ballesteros 1988, Bressan & Babbini 2003, Peña *et al.* 2015a). In agreement

with the predominance of sterile specimens typical of the main maerl-forming species reported in temperate Atlantic European beds (*P. calcareum, L. corallioides*), *P. lusitanicum* rarely showed reproductive structures in our collections. Nonetheless, we still were able to detect multiporate sporangial conceptacles in some collections of *P. lusitanicum* from southern Portugal (May, June and September) and from Ireland (September-November). In the literature, previous records of sexual uniporate conceptacles in crustose forms of *P. calcareum* (Cabioch 1970, Mendoza & Cabioch 1998) were recently confirmed with DNA barcodes (Peña *et al.* 2014b), which also suggested that vegetative multiplication by fragmentation could be the main mechanism of propagation in maerl beds.

Our molecular results that included the lectotype of *P. lamii* and the paratype of *P. laevigatum* allowed us to delimit *P. lusitanicum* by an integrative systematic approach. The three species shared a common external morphology of multiporate asexual conceptacles. However, *P. lamii* and *P. laevigatum* have never been reported as maerl-forming taxa (Adey & Adey 1973, Chamberlain 1991, Chamberlain & Irvine 1994). The sequence from the paratype of *P. laevigatum* was resolved as closely related to recent collections from Ireland and Atlantic coasts of France and Spain. This species was described from Helgoland (Germany), and has been reported as epilithic crusts that have multiporate sporangial conceptacles with a thickened rim, somewhat raised, and pore plate flush with surface, sunken or slightly raised, up to *ca.* 5 cells thick (Chamberlain & Irvine 1994). The present study also confirms literature records of *P. lamii* in intertidal and subtidal rocky shores along the Atlantic Iberian Peninsula (Ardré 1970, Chamberlain & Irvine 1994), being Algarve and Cádiz the southernmost localities. To date, *P. lamii* has been found only epilithic as a crustose growth-form, even in locations where it was found in maerl beds. Moreover, this is the first time that *P. lamii* is reported for subtidal maerl in Brittany (Adey & Adey 1973, Chamberlain 1991, Chamberlain & Irvine 1994). Finally, the comparison of our sequences from the type material of *P. lamii* with one specimen from NW Atlantic identified as *P. rugulosum* (voucher US170942) does not support the synonymization of these species proposed in Chamberlain (1991). *Phymatolithon rugulosum* was described by Adey (1964) in the Gulf of Maine, the same locality of our collection, but DNA sequences from its type material will be needed to draw more definitive conclusions.

Our phylogenetic analyses resolved the new maerl species *P. lusitanicum* and the crustose *P. lamii* as a sister taxon with full support, indicating that both species share a very recent common ancestor. Furthermore, the presence of crustose *P. lamii* in subtidal maerl beds of Brittany suggests that the speciation event is correlated to two clearly delimited

growth forms able to live under similar ecological conditions. Moreover, *P. lusitanicum* and *P. lamii* were resolved in a separate clade distantly related to other European *Phymatolithon* species that included the generitype *P. calcareum*.

An additional result derived from our molecular analyses is that *P. calcareum* and *Lithothamnion hamelii* must be regarded as conspecific. Importantly, this conclusion is based on sequence information obtained from neotype and lectotype material, respectively. Lemoine (1931) described *L. hamelii* but the lectotype material was designated more recently by Woelkerling & Lamy (1998). According to the priority principle of the International Code of Nomenclature (Article 11, ICN 2012), we propose *L. hamelii* as a heterotypic synonym of *P. calcareum.* Contrary to the common habit of *P. calcareum* as maerl, the lectotype of *L. hamelii* was an epilithic, crustose specimen collected by dredge in La Rance, Saint Servan (France) by Lami in 1930 (Woelkerling & Lamy 1998). Apart from *L. hamelii*, Lemoine (1931) reported other Melobesioideae species in Saint Servan such as *P. calcareum*, also subtidally in the same locality.

Finally, our COI-5P analyses revealed that *Phymatolithon* contains a high diversity of species in Atlantic European coasts with up to 10 supported lineages, while only seven are reported in the literature (Guiry & Guiry 2015). In addition, the analyses of COI-5P and *psb*A resolved several lineages for which specimens were not identified up to the species level, while other lineages encompassed specimens assigned to different species name. Further efforts in sequencing type material from other European species that failed in the present study (*Melobesia lenormandii* and *Lithothamnion purpureum*) will be required to provide an appropriate taxonomic baseline to clarify their identity. Alternatively, recent topotype material could be selected, but it should be studied with caution given the frequent co-occurrence of several *Phymatolithon* species in the same habitat. This observation has been pointed out in the literature (Chamberlain & Irvine 1994), and has been confirmed in the present study where specimens collected in the intertidal of the type locality of *P. purpureum* belonged to *P. lamii*. The presence of different clades with the name *P. lenormandii* and the absence of a DNA sequence from the type material cannot guaranty that the actual species is represented in our phylogeny. In addition, collections identified as *P. lenormandii* from Canada and *P. repandum* from New Zealand were resolved unrelated with other *Phymatolithon* taxa that, importantly, comprised sequences derived from type material for the generitype (*P. calcareum*). Hence, the inclusion of these taxa in our data set strongly suggests that the current concept and definition

of the genus *Phymatolithon* and its relationship with related genera (e.g. *Lithothamnion, Mesophyllum*) requires further evaluation.

Our understanding of the diversity of non-geniculate coralline algae in Europe has been much improved in the last five years by new DNA and morpho-anatomical data; as a result, two new species are now added as maerl-forming: *Phymatolithon lusitanicum* and *Mesophyllum sphaericum.* Previous reports that describe *P. calcareum* and *Lithothamnion corallioides* as the major builders of maerl beds in Europe need to be updated in both European and national legislation in order to ensure appropriate protection of these sensitive biogenic habitats.

SUPPLEMENTAL DATA

Table S1. Sample information for species included in molecular analyses. Type material in bold: holotype and isotype specimens of *Phymatolithon lusitanicum,* lectotypes of *Lithophyllum lamii* and *Lithothamnion hamelii,* paratype of *Lithothamnion laevigatum,* and neotype of *Phymatolithon calcareum.* Additional COI-5P and *psb*A sequences downloaded from GenBank are detailed.

Species	Voucher	GenBank accession number (COI-5P)	GenBank accession number (*psb*A)	Collection details	Herbarium
***Phymatolithon lusitanicum* sp. nov.(holotype)**	**CPVP-676**	**KC861627**	**KC819260**	**Subtidal (5 m), maerl, Con de Pego, Ría de Vigo, Galicia, Spain, 05/04/2011. Coll: Bárbara, I., Barreiro, R., Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.**	**SANT-Algae 29522**
***P. lusitanicum* sp. nov. (isotype)**	**CPVP-689**	**KC861620**	**-**	**Subtidal (5 m), maerl, Con de Pego, Ría de Vigo, Galicia, Spain, 05/04/2011. Coll: Bárbara, I., Barreiro, R., Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.**	**SANT-Algae 29523**
***P. lusitanicum* sp. nov. (isotype)**	**CPVP-685**	**KC861621**	**-**	**Subtidal (5 m), maerl, Con de Pego, Ría de Vigo, Galicia, Spain, 05/04/2011. Coll: Bárbara, I., Barreiro, R., Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.**	**SANT-Algae 29524**
P. lusitanicum	CPVP-1134	KC861646	-	Subtidal (5 m), maerl, Punta Barbafeita, Ría de Arousa, Galicia, Spain, 23/06/2011. Coll:Bárbara, I., Bunker, F. & Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29518
P. lusitanicum	CPVP- 1064	KC861649	-	Subtidal (5 m), maerl, Punta Barbafeita, Ría de Arousa, Galicia, Spain, 23/06/2011. Coll:Bárbara, I., Bunker, F. & Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29520
P. lusitanicum	CPVP-1060	KC861644	-	Subtidal (5 m), maerl, Punta Barbafeita, Ría de Arousa, Galicia, Spain, 23/06/2011. Coll:Bárbara, I., Bunker, F. & Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29521
P. lusitanicum	CPVP-1136	KC861645	-	Subtidal (5 m), maerl, Punta Barbafeita, Ría de Arousa, Galicia, Spain, 23/06/2011. Coll:Bárbara, I., Bunker, F. & Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29519
P. lusitanicum	CPVP-1261	KC861650	-	Subtidal (5 m), maerl, Sálvora, Ría de Arousa, Galicia, Spain, 05/08/2011. Coll: Bárbara, I., García, V. & Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29546
P. lusitanicum	CPVP-1260	KC861651	-	Subtidal (5 m), maerl, Sálvora, Ría de Arousa, Galicia, Spain, 05/08/2011. Coll: Bárbara, I., García, V. & Peña, V. Published in Pardo *et al.* (2014a)as *Phymatolithon* sp.3.	SANT-Algae 29545
P. lusitanicum	CPVP-1259	KC861652	-	Subtidal (5 m), maerl, Sálvora, Ría de Arousa, Galicia, Spain, 05/08/2011. Coll: Bárbara, I., García, V. & Peña, V. Published in Pardo *et al.* (2014a)as *Phymatolithon* sp.3.	SANT-Algae 29544
P. lusitanicum	CPVP-1258	KC861653	-	Subtidal (5 m), maerl, Sálvora, Ría de Arousa, Galicia, Spain, 05/08/2011. Coll: Bárbara, I., García, V. & Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29543

P. lusitanicum	CPVP-1257	KC861654	-	Subtidal (5 m), maerl, Sálvora, Ría de Arousa, Galicia, Spain, 05/08/2011. Coll: Bárbara, I., García, V. & Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3	SANT-Algae 29542
P. lusitanicum	CPVP-1256	KC861655	-	Subtidal (5 m), maerl, Sálvora, Ría de Arousa, Galicia, Spain, 05/08/2011. Coll: Bárbara, I., García, V. & Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29541
P. lusitanicum	CPVP-633	KC861660	-	Subtidal (9 m), maerl, Playa de Tulla, Ría de Pontevedra, Galicia, Spain, 07/04/2011. Coll: Bárbara, I., Barreiro, R., Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29548
P. lusitanicum	CPVP-639	KC861642	KC819258	Subtidal (9 m), maerl, Playa de Tulla, Ría de Pontevedra, Galicia, Spain, 07/04/2011. Coll: Bárbara, I., Barreiro, R., Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29547
P. lusitanicum	CPVP-626	KC861662	-	Subtidal (9 m), maerl, Playa de Tulla, Ría de Pontevedra, Galicia, Spain, 07/04/2011. Coll: Bárbara, I., Barreiro, R., Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29550
P. lusitanicum	CPVP-627	KC861617	-	Subtidal (9 m), maerl, Playa de Tulla, Ría de Pontevedra, Galicia, Spain, 07/04/2011. Coll: Bárbara, I., Barreiro, R., Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29552
P. lusitanicum	CPVP-622	KC861628	-	Subtidal (9 m), maerl, Playa de Tulla, Ría de Pontevedra, Galicia, Spain, 07/04/2011. Coll: Bárbara, I., Barreiro, R., Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29549
P. lusitanicum	CPVP-618	KC861641	-	Subtidal (9 m), maerl, Playa de Tulla, Ría de Pontevedra, Galicia, Spain, 07/04/2011. Coll: Bárbara, I., Barreiro, R., Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29551
P. lusitanicum	CPVP-611	KC861647	-	Subtidal (13 m), maerl, Isla Ons, Ría de Pontevedra Galicia, Spain, 07/04/2011. Coll: Bárbara, I., Barreiro, R., Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29533
P. lusitanicum	CPVP-600	KC861648	-	Subtidal (13 m), maerl, Isla Ons, Ría de Pontevedra Galicia, Spain, 07/04/2011. Coll: Bárbara, I., Barreiro, R., Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29534
P. lusitanicum	CPVP-749	KC861656	-	Subtidal (5 m), maerl, Baliza Tofiño, Ría de Vigo, Galicia, Spain, 05/04/2011, coll: Barreiro, R., Bárbara, I. & Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29532
P. lusitanicum	CPVP-748	KC861657	-	Subtidal (5 m), maerl, Baliza Tofiño, Ría de Vigo, Galicia, Spain, 05/04/2011, coll: Barreiro, R., Bárbara, I. & Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3	SANT-Algae 29531
P. lusitanicum	CPVP-50	KC861659	-	Subtidal (5 m), maerl, Baliza Tofiño, Ría de Vigo, Galicia, Spain, 19/05/2009, coll: Bárbara, I. & Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29530
P. lusitanicum	CPVP-686	KC861622	-	Subtidal (5 m), maerl, Con de Pego, Ría de Vigo, Galicia, Spain, 05/04/2011. Coll: Bárbara, I., Barreiro, R., Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29529
P. lusitanicum	CPVP-681	KC861623	-	Subtidal (5 m), maerl, Con de Pego, Ría de Vigo, Galicia, Spain, 05/04/2011. Coll: Bárbara, I., Barreiro, R., Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29528
P. lusitanicum	CPVP-680	KC861624	-	Subtidal (5 m), maerl, Con de Pego, Ría de Vigo, Galicia, Spain, 05/04/2011. Coll: Bárbara, I., Barreiro, R., Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29527

P. lusitanicum	CPVP-695	KC861625	-	Subtidal (5 m), maerl, Con de Pego, Ría de Vigo, Galicia, Spain, 05/04/2011. Coll: Bárbara, I., Barreiro, R., Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29526
P. lusitanicum	CPVP-694	KC867626	-	Subtidal (5 m), maerl, Con de Pego, Ría de Vigo, Galicia, Spain, 05/04/2011. Coll: Bárbara, I., Barreiro, R., Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29525
P. lusitanicum	CPVP-644	KC861635	-	Subtidal (11 m), maerl, Islas Cíes, Ria de Vigo, Galicia, 05/04/2011. Coll: Bárbara, I., Barreiro, R., Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3	SANT-Algae 29538
P. lusitanicum	CPVP-645	KC861636	-	Subtidal (11 m), maerl, Islas Cíes, Ria de Vigo, Galicia, 05/04/2011. Coll: Bárbara, I., Barreiro, R., Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29536
P. lusitanicum	CPVP-646	KC861637	-	Subtidal (11 m), maerl, Islas Cíes, Ria de Vigo, Galicia, 05/04/2011. Coll: Bárbara, I., Barreiro, R., Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29535
P. lusitanicum	CPVP-670	KC861638	-	Subtidal (11 m), maerl, Islas Cíes, Ria de Vigo, Galicia, 05/04/2011. Coll: Bárbara, I., Barreiro, R., Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29537
P. lusitanicum	CPVP-664	KC861639	-	Subtidal (11 m), maerl, Islas Cíes, Ria de Vigo, Galicia, 05/04/2011. Coll: Bárbara, I., Barreiro, R., Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29540
P. lusitanicum	CPVP-669	KC861640	-	Subtidal (11 m), maerl, Islas Cíes, Ria de Vigo, Galicia, 05/04/2011. Coll: Bárbara, I., Barreiro, R., Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29539
P. lusitanicum	CPVP-77	KC861643	-	Subtidal (15 m), maerl, Lagos, Algarve, Portugal, 04/09/2008. Coll: Bárbara, I. & Peña, V. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29553
P. lusitanicum	CPVP-453	KC861632	-	Subtidal (20 m), maerl, Armaçao de Pêra, Algarve, Portugal, 02/03/2011. Coll: Neves, P. & Rodrigues, M. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29516
P. lusitanicum	CPVP-451	KC861631	-	Subtidal (20 m), maerl, Armaçao de Pêra, Algarve, Portugal, 02/03/2011. Coll: Neves, P. & Rodrigues, M. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29515
P. lusitanicum	CPVP-452	KC861618	-	Subtidal (20 m), maerl, Armaçao de Pêra, Algarve, Portugal, 02/03/2011. Coll: Neves, P. & Rodrigues, M. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29514
P. lusitanicum	CPVP-478	KC861630	KC819253	Subtidal (20 m), maerl, Armaçao de Pêra, Algarve, Portugal, 02/03/2011. Coll: Neves, P. & Rodrigues, M. Published in Pardo *et al.* (2014a) as Phymatolithon sp.3.	SANT-Algae 29517
P. lusitanicum	CPVP-503	KC861633	-	Subtidal (20 m), maerl, Armaçao de Pêra, Algarve, Portugal, 02/03/2011. Coll: Neves, P. & Rodrigues, M. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29511
P. lusitanicum	CPVP-480	KC861629	-	Subtidal (20 m), maerl, Armaçao de Pêra, Algarve, Portugal, 02/03/2011. Coll: Neves, P. & Rodrigues, M. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29513
P. lusitanicum	CPVP-501	KC861661	-	Subtidal (20 m), maerl, Armaçao de Pêra, Algarve, Portugal, 23/02/2011. Coll: Neves, P. & Rodrigues, M. Published in Pardo *et al.* (2014a) as *Phymatolithon* sp.3.	SANT-Algae 29512

P. lusitanicum	VPF00511	KT807920	-	Subtidal (40-44 m), maerl, Alborán Sea, Mediterranean Spain, 22/09/2011. Coll: INDEMARES project.	SANT-Algae 29508
P. lusitanicum	VPF00451	KT807910	-	Subtidal (42-48 m), maerl, Alborán Sea, Mediterranean Spain, 24/09/2011. Coll: INDEMARES project.	SANT-Algae 29506
P. lusitanicum	E113	-	KT874588	Subtidal (6 m), maerl, Carraroe beach, Ireland, 09/05/2009. Coll.: Hernández, J. & Hanniffy, D. Published in Hernández-Kantún *et al.* (2014) as *P. purpureum.*	GALW015741
P. lusitanicum	E17	-	KT863473	Subtidal (6 m), maerl, Carraroe beach, Ireland, 08/10/2009. Coll.: Moriarty, M.	SANT-Algae 29595
P. lusitanicum	E10	-	KT863474	Subtidal (6 m), maerl, Carraroe beach, Ireland, 08/10/2009. Coll.: Moriarty, M.	SANT-Algae 29596
P. lusitanicum	E302	-	KT863475	Intertidal, maerl, Mukinish, Ireland, 04/11/2009. Coll.: Hernández, J.	SANT-Algae 29597
Phymatolithon cf. brunneum Y.M.Chamberlain	VPF00561B	KT807916	-	Intertidal, epilithic crust, Laredo-Puerto Viejo, Cantabria, Spain, 22/02/2011. Coll: Secilla, A. & Peña, V.	SANT-Algae 29488
P. cf. brunneum	VPF00576	KT807908	-	Intertidal, epilithic crust, San Juan de Gaztelugatxe, Pais Vasco, Spain, 19/02/2011. Coll: Secilla, A. & Peña, V.	SANT-Algae 29503
P. cf. brunneum	VPF00572	KT807909	KT807938	Intertidal, epilithic crust, San Juan de Gaztelugatxe, Pais Vasco, Spain, 19/02/2011. Coll: Secilla, A. & Peña, V.	SANT-Algae 29502
Phymatolithon calcareum (Pallas) Adey & McKibbin	VPF00132	KT807904	-	Subtidal (< 10 m), crust in maerl bed, Molène, Brittany, 13/05/2011. Coll: Grall, J. & Peña, V.	SANT-Algae 29484
P. calcareum	VPF00276	KT807928	-	Subtidal (5-10 m), maerl, Osundet, Hordaland, Norway, nd. Coll: Rueness, J.	SANT-Algae 29487
P. calcareum	VPF00523	KT807921	-	Subtidal (25 m), maerl, Alborán Sea, Mediterranean Spain, 25/09/2011. Coll: INDEMARES project	SANT-Algae 29505
P. calcareum	VPF00449	KT807905	-	Subtidal (42-48 m), maerl, Alborán Sea, Mediterranean Spain, 24/09/2011. Coll: INDEMARES project	SANT-Algae 29507
***Lithothamnion hamelii* Me. Lemoine (lectotype)**	**PC0145174**	**-**	**KT807946**	**Subtidal (8 m), crust, St Servan, Pointe de Cancaval, Rance, France, 1930. Coll: Lami, R.**	**PC0145174**
***Phymatolithon laevigatum* (Foslie) Foslie [*Lithothamnion laevigatum* Foslie] (paratype)**	**PC0145173**	**-**	**KT807943**	**Crust, Helgoland, Germany, 29/01/1894. Coll: Kuckuck, P.**	**PC0145173**
P. cf. laevigatum	VPF00033	KT807901	KT807937	Intertidal, epilithic crust, Camelle, Galicia, Spain, 31/08/2011. Coll: Peña, V.	SANT-Algae 29496
P. cf. laevigatum	VPF00014	KT807927	-	Subtidal (9-11 m), epilithic crust, Ziérbena, Puerto Bilbao, Pais Vasco, Spain, 14/07/2011. Coll: Santolaria, A.	SANT-Algae 29479
P. cf. laevigatum	VPF00383C	KT807925	-	Intertidal, epilithic crust, Montedor, Portugal, 18/04/2011. Coll: Secilla, A. & Peña, V.	SANT-Algae 29497

P. cf. laevigatum	VPF00574	KT807918	-	Intertidal, epilithic crust, San Juan de Gaztelugatxe, Pais Vasco, Spain, 19/02/2011. Coll: Secilla, A. & Peña, V.	SANT-Algae 29504
***Phymatolithon lamii* (Me.Lemoine) Y.M.Chamberlain [*Lithophyllum lamii* Me. Lemoine] (lectotype)**	**PC0719024**	-	**KT807939**	**Subtidal (8 m), crust, St Servan, Pointe de Cancaval, Rance, France, 1930. Coll: Lami, R.**	**PC0719024**
P. lamii	VPF00557	KT807915	-	Intertidal, epilithic crust, Sainte-Marguerite, Landeda, Brittany, France, 19/03/2011. Coll: Peña, V.	SANT-Algae 29480
P. lamii	VPF00410	KT807923	-	Subtidal (< 10 m), epilithic crust in maerl bed, Molène, Brittany, 13/05/2011. Coll: Grall, J. & Peña, V.	SANT-Algae 29485
P.lamii	VPF00223	KT807914	KT807942	Intertidal, epilithic crust, Fort du Mingant, Brest, Brittany, France, 18/03/2011. Coll: Peña, V.	SANT-Algae 29491
P. lamii	VPF00222	KT807924	-	Intertidal, epilithic crust, Fort du Mingant, Brest, Brittany, France, 18/03/2011. Coll: Peña, V.	SANT-Algae 29493
P. lamii	VPF00225	KT807907	-	Intertidal, epilithic crust, Fort du Mingant, Brest, Brittany, France, 18/03/2011. Coll: Peña, V.	SANT-Algae 29489
P. lamii	VPF00236	KT807926	-	Intertidal, epilithic crust, Fort du Mingant, Brest, Brittany, France, 18/03/2011. Coll: Peña, V.	SANT-Algae 29495
P. lamii	VPF00088	KT807902	-	Subtidal (7 m), epilithic crust, Astondo, Bizkaia, Pais Vasco, Spain, 18/07/2011. Coll: Santolaria, A.	SANT-Algae 29501
P. lamii	VPF00561A	KT807903	-	Intertidal, epilithic crust, Laredo-Puerto Viejo, Cantabria, Spain, 22/02/2011. Coll: Secilla, A. & Peña, V.	SANT-Algae 29488
P. lamii	VPF00074	KT807917	-	Intertidal pool, epilithic crust, Cadavedo, Asturias, Spain, 03/08/2011. Coll: Peña, V.	SANT-Algae 29499
P. lamii	VPF00075	KT807906	-	Intertidal pool, epilithic crust, Cadavedo, Asturias, Spain, 03/08/2011. Coll: Peña, V.	SANT-Algae 29498
P. lamii	VPF00369B	-	KT807947	Subtidal (2 m), epilithic crust, Peinzás, Galicia, Spain, 09/02/2011. Coll: Pardo, C., Bárbara, I, Maneiro, I., Peña, V.	SANT-Algae 29118
P. lamii	VPF00320	KT807919	-	Intertidal, epilithic crust, Cala Encendida, Cadiz, Spain, 18/02/2011. Coll: Díaz, P. & Bárbara, I.	SANT-Algae 29478
P. lamii	VPF00325	KT807912	KT807941	Intertidal, epilithic crust, Cala Encendida, Cadiz, Spain, 18/02/2011. Coll: Díaz, P. & Bárbara, I.	SANT-Algae 29477
Phymatolithon cf. lenormandii (Areschoug) Adey	VPF00081	KT807911	KT807940	Intertidal pool, epilithic crust, Cadavedo, Asturias, Spain, 03/08/2011. Coll: Peña, V.	SANT-Algae 29500
P. lenormandii	US 170885	KT863470	KP142718	Intertidal, epilithic crust, Nichol Is, off Ship Harbour Nova Scotia, Canada, 3/10/1964. Coll: Adey, W.H.	US 170885

P. lenormandii	US 170941	KT863471	KP142719	Subtidal (0-3 m), epilithic crust, Samlenfjord, Hardangerfjord, Norway, 31/07/1967. Coll: Adey, W.H.	US 170941
Phymatolithon rugulosum W.H.Adey	US 170942	KT863472	KP142717	Subtidal (3-8 m), epilithic crust, Schoodic pt., ME, Gulf of Maine, US, 26/06/1962. Coll: Adey, W.H.	US170942
Phymatolithon sp.	VPF00425A	-	KT807945	Subtidal (11 m), epilithic crust in maerl bed, Islas Cíes, Ria de Vigo, Galicia, 05/04/2011. Coll: Bárbara, I., Barreiro, R., Peña, V.	SANT-Algae 29481
Phymatolithon sp.	VPF00432	KT807922	-	Subtidal (2 m), epilithic crust, Peinzás, Galicia, Spain, 25/02/2012. Coll: Pardo, C., Bárbara, I, Maneiro, I., Peña, V.	SANT-Algae 29476
Phymatolithon sp.	VPF00615A	KT807913	-	Subtidal, maerl, Douarnenez, Brittany, France, 22/08/2012. Coll: Le Gall, L.	PC0145175

Additional sequences analysed

Species	**Voucher**	**GenBank accession numbers (COI-5P/*psb*A)**	**Collection details**
***Phymatolithon calcareum* (Pallas) Adey &McKibbin (neotype)**	**000712373BM**	**KF808323/JQ89623**	**St Mawes Bank, Falmouth, England (Woelkerling & Irvine 1986, Pardo *et al.* 2014a, Peña *et al.* 2014b, Hernández-Kantún *et al.* 2015b)**
P. calcareum	CPVP-781	KC861616	Ireland, Pardo *et al.* (2014a)
P. calcareum	CPVP-157	KC861615	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-151	KC861614	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-135	KC861613	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-956	KC861612	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-130	KC861612	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-275	KC861611	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-899	KC861610	Brittany, France, Pardo *et al.* (2014a)
P. calcareum	CPVP-897	KC861609	Brittany, France, Pardo *et al.* (2014a)
P. calcareum	CPVP-906	KC861608	Brittany, France, Pardo *et al.* (2014a)
P. calcareum	CPVP-903	KC861607	Brittany, France, Pardo *et al.* (2014a)
P. calcareum	CPVP-909	KC861606	Brittany, France, Pardo *et al.* (2014a)
P. calcareum	CPVP-912	KC861605/KC819243	Brittany, France, Pardo *et al.* (2014a)
P. calcareum	CPVP-47	KC861604	Cornwall, England, UK, Pardo *et al.* (2014a)
P. calcareum	CPVP-696	KC861603	Galicia, Spain, Pardo *et al.* (2014a)

P. calcareum	CPVP-779	KC861602	Ireland, Pardo *et al.* (2014a)
P. calcareum	CPVP-654	KC861601	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-648	KC861600	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-665	KC861599	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-167	KC861598	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-766	KC861597	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-565	KC861596	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-566	KC861595	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-310	KC861594	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-554	KC861593	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-560	KC861592	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-955	KC861591	Brittany, France, Pardo *et al.* (2014a)
P. calcareum	CPVP-558	KC861590	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-916	KC861589	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-629	KC861587	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-628	KC861586	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-48	KC861585	England, UK, Pardo *et al.* (2014a)
P. calcareum	CPVP-207	KC861584	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-174	KC861583	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-1078	KC861582	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-1067	KC861581	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-1065	KC861580	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-1129	KC861579	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-1121	KC861578	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-1120	KC861577	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-1119	KC861576	Galicia, Spain, Pardo *et al.* (2014a)

P. calcareum	CPVP-1118	KC861575	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-1110	KC861574	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-1109	KC861573	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-1108	KC861572	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-1104	KC861571	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-1103	KC861570	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-655	KC861569	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-170	KC861568	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-164	KC861567	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-1104	KC861571	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-163	KC861566	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-162	KC861565	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-46	KC861564	England, UK, Pardo *et al.* (2014a)
P. calcareum	CPVP-783	KC861563	Ireland, Pardo *et al.* (2014a)
P. calcareum	CPVP-780	KC861562	Ireland, Pardo *et al.* (2014a)
P. calcareum	CPVP-778	KC861561	Ireland, Pardo *et al.* (2014a)
P. calcareum	CPVP-1195	KC861557	England, UK, Pardo *et al.* (2014a)
P. calcareum	CPVP-1187	KC861556	England, UK, Pardo *et al.* (2014a)
P. calcareum	CPVP-961	KC861555	Brittany, France, Pardo *et al.* (2014a)
P. calcareum	CPVP-959	KC861554	Brittany, France, Pardo *et al.* (2014a)
P. calcareum	CPVP-1234	KC861559	La Rochelle, France, Pardo *et al.* (2014a)
P. calcareum	CPVP-1236	KC861560	La Rochelle, France, Pardo *et al.* (2014a)
P. calcareum	CPVP-858	KC861553	Brittany, France, Pardo *et al.* (2014a)
P. calcareum	CPVP-615	KC861552	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-607	KC861551	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-603	KC861550	Galicia, Spain, Pardo *et al.* (2014a)

P. calcareum	CPVP-1450	KC861549	Norway, Pardo *et al.* (2014a)
P. calcareum	CPVP-44	KC861548	England, UK, Pardo *et al.* (2014a)
P. calcareum	CPVP-43	KC861547	England, UK, Pardo *et al.* (2014a)
P. calcareum	CPVP-753	KC861546	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	CPVP-929	KC861545	Brittany, France, Pardo *et al.* (2014a)
P. calcareum	CPVP-921	KC861544	Brittany, France, Pardo *et al.* (2014a)
P. calcareum	CPVP-920	KC861543	Brittany, France, Pardo *et al.* (2014a)
P. calcareum	CPVP-1254	KC861542	Northern Ireland, UK, Pardo *et al.* (2014a)
P. calcareum	CPVP-1253	KC861541	Northern Ireland, UK, Pardo *et al.* (2014a)
P. calcareum	CPVP-1252	KC861540	Northern Ireland, UK, Pardo *et al.* (2014a)
P. calcareum	CPVP-1251	KC861539	Northern Ireland, UK, Pardo *et al.* (2014a)
P. calcareum	CPVP-1247	KC861538	Northern Ireland, UK, Pardo *et al.* (2014a)
P. calcareum	CPVP-1246	KC861537	Northern Ireland, UK, Pardo *et al.* (2014a)
P. calcareum	CPVP-1245	KC861536	Northern Ireland, UK, Pardo *et al.* (2014a)
P. calcareum	CPVP-1244	KC861535	Northern Ireland, UK, Pardo *et al.* (2014a)
P. calcareum	CPVP-1243	KC861534	Northern Ireland, UK, Pardo *et al.* (2014a)
P. calcareum	CPVP-1242	KC861533	Northern Ireland, UK, Pardo *et al.* (2014a)
P. calcareum	CPVP-900	KC861532	Brittany, France, Pardo *et al.* (2014a)
P. calcareum	CPVP-901	KC861531	Brittany, France, Pardo *et al.* (2014a)
P. calcareum	CPVP-910	KC861530	Brittany, France, Pardo *et al.* (2014a)
P. calcareum	CPVP-943	KC861529	Brittany, France, Pardo *et al.* (2014a)
P. calcareum	CPVP-555	KC861528	Galicia, Spain, Pardo *et al.* (2014a)
P. calcareum	LBC0001	GQ917247/GQ917436	France, Bittner *et al.* (2010)
P. calcareum	CPVP-1188	KC861558	Milford Haven, UK, Pardo *et al.* (2014a)
Phymatolithon lamii	GALW15780	-/JQ896248	Ireland, Hernández-Kantún *et al.* (2015b)
Phymatolithon lenormandii	GWS005398	JX572005	Canada, Saunders & McDevit, unpublished

P. lenormandii	GWS005396	JX571978	Canada, Saunders & McDevit, unpublished
P. lenormandii	GALW15781	-/JQ896249	Ireland, Hernández-Kantún *et al.* (2015b)
Phymatolithon purpureum (P.L. Crouan & H.M. Crouan) Woelkerling & L.M.Irvine	GALW15782	-/JQ896232	Kingstown Bay, Ireland, Hernández-Kantún *et al.* (2015b)
Phymatolithon repandum (Foslie) Wilks & Woelkerling	ND114	-/FJ361450	New Zealand, Broom *et al.*, unpublished
P. repandum	ND175	-/FJ361477	New Zealand, Broom *et al.*, unpublished
P. repandum	ND659	-/FJ361683	New Zealand, Broom *et al.*, unpublished
Phymatolithon sp.	GALW15783	-/JQ896247	Ireland, Hernández-Kantún *et al.* (2015b)
Phymatolithon sp.2	CPVP-441	KC861668/KC819250	Madeira, Pardo *et al.* (2014a)
Phymatolithon sp.2	CPVP-440	KC861667	Madeira, Pardo *et al.* (2014a)
Phymatolithon sp.2	CPVP-443	KC861665/KC819251	Madeira, Pardo *et al.* (2014a)
Phymatolithon sp.1	CPVP-510	KC861664/KC819255	Algarve, Portugal, Pardo *et al.* (2014a)
Phymatolithon sp.1	CPVP-868	KC861663	Brittany, France, Pardo *et al.* (2014a)
Uncultured Corallinales	LBC0028	GQ917512/GQ917711	France, Bittner *et al.* (2010)
Uncultured Corallinales	LBC0008	-/GQ917707	France, Bittner *et al.* (2010)
Uncultured Corallinales	LBC0013	-/GQ917708	France, Bittner *et al.* (2010)
Unidentified Hapalidiaceae	LBC0005	-/GQ917437	France, Bittner *et al.* (2010)
Lithothamnion corallioides (P.L.Crouan & H.M.Crouan) P.L.Crouan & H.M.Crouan	CPVP-817	KC861448/KC819265	Brittany, France, Pardo *et al.* (2014a)
L. corallioides	CPVP-1238	KC861497	La Rochelle, France, Pardo *et al.* (2014a)
Lithothamnion glaciale Kjellman	GWS007542	HM918812/JQ422235	Newfoundland and Labrador, Canada, Le Gall *et al.,* unpublished
Mesophyllum cf. *erubescens* (Foslie) Me.Lemoine	LBC0551	GQ917256/GQ917446	Vanuatu, Bittner *et al.* (2010)
M. lichenoides (J.Ellis) Me.Lemoine	LBC0031	GQ917249/GQ917439	Atlantic France, Bittner *et al.* (2010)
M. *sphaericum* V.Peña, Bárbara, W.H.Adey, Riosmena-Rodriguez & H.G.Choi (holotype)	CPVP-776	KC861526/KC819262	Galicia, Spain, Pardo *et al.* (2014a)

CHAPTER 3

Detection of gametophytes in thte maerl-forming species *Phymatolithon calcareum* (Melobesioideae, Rhodophyta) assessed by DNA barcoding

This chapter is based on content published in:

Peña V., Hernández-Kantún J. J., Grall J., Pardo C., López L., Bárbara I., Le Gall L. & Barreiro R. (2014). Detection of gametophytes in the maerl-forming species Phymatolithon calcareum (Melobesioideae, Corallinales) assessed by DNA barcoding. Cryptogamie, Algologie 35, 15-25.

DETECTION OF GAMETOPHYTES IN THE MAERL-FORMING SPECIES *PHYMATOLITHON CALCAREUM* (MELOBESIOIDEAE, RHODOPHYTA) ASSESSED BY DNA BARCODING

ABSTRACT

Fertile gametangial plants of *Phymatolithon calcareum*, which are seldom reported in the Atlantic European coasts, were collected as encrusting, epilithic plants in a subtidal maerl bed in Brittany (France). Based on their morphological features, the plants were identified as *P. calcareum*. This identification was further confirmed by DNA barcodes using as a reference COI-5P sequences obtained from the neotype together with recent collections from the Atlantic European maerl beds. The reproductive structures were empty but they were regarded as mature female conceptacles. Compared to the two previous records of gametangial plants of *P. calcareum* for the Atlantic European waters, the uniporate conceptacles observed in this study are larger, and were collected at a different time of the year. To our knowledge, this is the first time that the occurrence of gametangial plants of *P. calcareum* is corroborated with molecular tools (DNA barcodes).

KEYWORDS: COI-5P, crustose coralline algae, European coast, reproduction, systematics.

INTRODUCCTION

Maerl beds are deposits of unattached, non-geniculate coralline red algae with a variable branching density that provides a wide range of ecological niches (Birkett *et al.* 1998, BIOMAERL Team 2003). In the North East Atlantic, they are known from Arctic regions in Iceland and Svalbard Archipelago (Adey 1968, Gunnarsson 1977, Teichert *et al.* 2012) to subtropical and tropical archipelagos in the Macaronesia (Foslie 1908, Cabioch 1974, Afonso-Carrillo & Gil-Rodríguez 1982). This latitudinal gradient entails changes in the species composition of maerl beds and the replacement of the maerl-forming species (Grall 2003, Hall-Spencer *et al.* 2010), being *Phymatolithon calcareum* (Pallas) W.H. Adey & D.L. McKibbin one of the species most commonly cited in the Atlantic European maerl beds (Hall-Spencer et al. 2010).

Although thallus fragmentation is suggested as the major mechanism of propagation where extensive unattached populations occur (Bosence 1976, Johansen 1981), fertile unattached plants have also been found in European maerl beds. Multiporate sporangial conceptacles of *P. calcareum* have been observed by several authors on unattached specimens in maerl beds of the Atlantic European coast (Lemoine 1910, Suneson 1958, Cabioch 1969, Adey & McKibbin 1970, Woelkerling & Irvine 1986, Irvine & Chamberlain 1994, Peña & Bárbara 2004, 2008b). In comparison, uniporate gametangial conceptacles were reported only very rarely on small encrusting plants attached to gravel and dead maerl in a subtidal bed in Brittany (Mendoza & Cabioch 1998). Previously, Suneson (1958) reported the occurrence of female conceptacles containing ripe carposporangia in material collected from this region, but the author did not provide further details. By contrast, fertile gametangial and sporangial plants of *P. calcareum* are common in the Mediterranean maerl beds (Bressan & Babbini 2003). In Alaska, uniporate male conceptacles have been reported in unattached plants of *P. calcareum* (Konar *et al.* 2006).

Identifying coralline species based on morphology is challenging due to the high morphological plasticity of the group (Steneck 1986). In unattached coralline plants, morphological variation can be extreme even within a single species, and it is likely related to local hydrodynamic conditions (Bosence 1976, Steller & Foster 1995, Schaeffer *et al.* 2002, Basso *et al.* 2009, Peña & Bárbara 2009). Fortunately, recent assessments of species diversity has benefited from the recent advent of DNA barcodes to the taxonomist's toolbox. This technique uses sequences of a fragment of the mitochondrial cytochrome c oxidase subunit 1

gene (COI-5P) for species recognition. DNA barcodes are now well established in many red algal groups (e.g. Saunders 2005, Le Gall & Saunders 2010b) and they have been successfully applied to the identification of coralline species and to the detection of cryptic taxa (Walker *et al.* 2009, Bittner *et al.* 2011, Hind & Saunders 2013a). In the particular case of maerl-forming species, DNA barcodes are used in an on-going Barcode of Life Database (BOLD, http:// www.boldsystems.org/) project called ''maerl-NE Atlantic''(code MAERL) that focuses on the assessment of the diversity of maerl species along the Atlantic European coasts (Pardo *et al.* 2012, 2014a). The project promoted extensive collections of maerl and associated encrusting corallines by SCUBA diving and dredging in maerl beds scattered along the study area. As part of the project, COI-5P sequences were produced for new collections of *P. calcareum* from maerl beds of Brittany, Britain, Ireland, and Galicia; these collections included material from the neotype locality (Falmouth, Cornwall, UK, Woelkerling & Irvine 1986). Moreover, our identification of *P. calcareum* is supported by molecular information obtained from the neotype material deposited in the British Museum of Natural History (herbarium BM, London).

As part of one collection from a Breton subtidal maerl bed, we found uniporate conceptacles in two epilithic, encrusting branched plants. Both specimens were preliminary identified as *P. calcareum* based on their morphological features. However the scarcity of records with sexual uniporate conceptacles for this species in Atlantic maerl beds advised that a deeper examination was required to provide further evidence in support of the species recognition. The aforementioned availability of COI-5P sequences for this species offered and excellent opportunity to obtain this corroboration.

MATERIAL AND METHODS

Samples were collected by dredging in the maerl bed of Molène Archipelago (Finistère, Brittany, France) in May 2011 at 3-7 m depth (**Fig 1**, **Table 1**). Fertile encrusting attached specimens *of Phymatolithon calcareum* with uniporate conceptacles were selected for anatomical and molecular examination (CPVP-943 and CPVP-955, **Table 1**). They were air-dried and preserved in zipper bags with silica gel. For the anatomical study, selected branches of the two specimens with uniporate conceptacles were examined under Scanning Electron Microscope (SEM, model JEOL JSM 6400, Universidade da Coruña, Spain). The specimens were deposited in the Herbarium SANT Algae (Universidade de Santiago de Compostela, Spain).

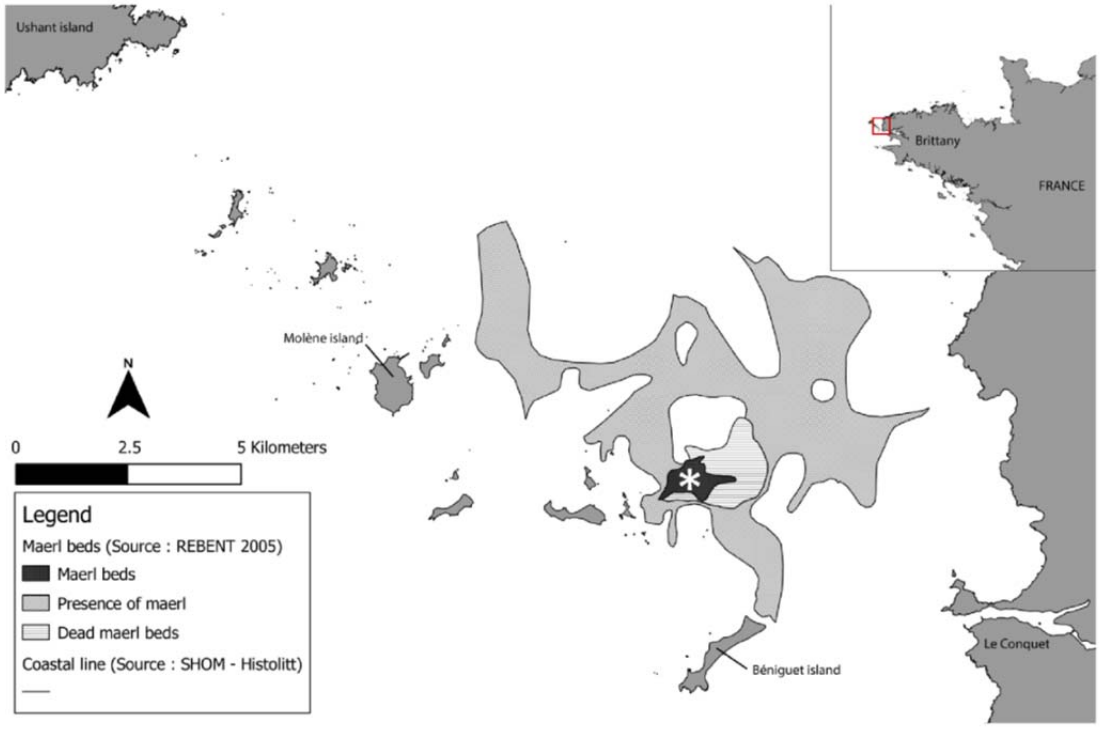


Fig. 1. Location of the maerl bed in Molène Archipelago (Finistère, Brittany, France) and collection area (black area marked with asterisk). Resource: IFREMER.

For the molecular study, the specimens were cleaned under stereomicroscope and clean surfaces were ground with an electric drill bite. Genomic DNA was extracted using DNeasy® Blood & Tissue Kit (Qiagen Inc., Valencia, California USA) following the manufacturer's recommendations with minor modifications. A fragment of the 5' end of the COI gene (COI-5P) was PCR-amplified with the primers GazF1 and GazR1 (Saunders 2005). PCR amplification followed Saunders & McDevit (2012b). PCR products were purified with exonuclease I and shrimp alkaline phosphatas –ExoSap-(Fermentas, Thermo Fisher Scientific, Spain), and sequenced at Macrogen facilities (http://www.macrogen.com). Sequences were submitted to the on-going BOLD (project ''maerl-NE Atlantic", code MAERL, http://www.boldsystems.org/) and GenBank database (**Table 1**). They were compared with 17 COI-5P sequences of *P. calcareum* obtained from the neotype (BM Box 1626, see **Fig. 2 in chapter 1**, Woelkerling & Irvine 1986) as well as from recent collections from the neotype locality (Falmouth, Cornwall, UK) and from other Atlantic maerl beds of Britain, Ireland, Galicia and Brittany (**Table 1**). Collections from Brittany included the locality where the gametophytes were previously recorded (Guerheon, Brittany, Mendoza & Cabioch 1998, **Table 1**). COI-5P sequences were also obtained for two other maerl species *Lithothamnion corallioides* (P.L. Crouan & H.M. Crouan)

Table 1. Sample information for the collections included in the DNA barcode analyses. BOLD Process IDs and GenBank accession numbers are provided for the specimens. Underlined text: neotype of *Phymatolithon calcareum* and holotype of *Mesophyllum sphaericum*. Bold text: gametophytes of *P. calcareum* detected in this study.

Species	Collection details	Voucher	BOLD Process IDs	GenBank accession no.
Phymatolithon calcareum (Pallas) W.H. Adey & D.L. McKibbin	St. Mawes Bank, Falmouth Harbour, Cornwall, UK, 11/12/1983. Coll: W.F. Farnham. Neotype material.	BM000712373	MAERL237-13	KF808323
Phymatolithon calcareum	St. Mawes Bank, Falmouth Harbour, Cornwall, UK, -18 m, 02/06/2010. Coll: J.M. Hall-Spencer.	CPVP-47	MAERL054-11	KC861604
Phymatolithon calcareum	Milford Haven, Wales, UK, -4 m, 14/07/2011. Coll: F. Bunker	CPVP-1188	MAERL124-13	KC861558
Phymatolithon calcareum	Zara Shoal, Strangford Lough, Northern Ireland, -10 m, 25/07/2011. Coll: L. Kregting, D. Pritchard	CPVP-1243	MAERL148-13	KC861534
Phymatolithon calcareum	Gleesk Pier, Co. Kerry, Ireland, -10 m, June 2008. Coll: M. Moriarty.	CPVP-778	MAERL121-13	KC861561
Phymatolithon calcareum	Carraroe, Co. Galway, Ireland, -5 m, 21/10/2010. Coll: N. Nolan, J. Hernandez	CPVP-781	MAERL066-11	KC861616
Phymatolithon calcareum	Guerheon, Baie de Morlaix, Brittany, -11 m, 10/05/2011. Coll: T. Wilfried, Y. Fontana	CPVP-906	MAERL058-11	KC861608
Phymatolithon calcareum	Molène Archipelago, Finistère, Brittany, -10 m, 10/03/2011. Coll: V. Peña	CPVP-858	MAERL129-13	KC861553
Phymatolithon calcareum	Molène Archipelago, Finistère, Brittany, -10 m, 10/05/2011. Coll: J. Grall, V. Peña. **Uniporate conceptacles.**	**CPVP-955**	MAERL236-13	KC861591
Phymatolithon calcareum	Molène Archipelago, Finistère, Brittany, -10 m, 10/05/2011. Coll: J. Grall, V. Peña. **Uniporate conceptacles.**	**CPVP-943**	MAERL235-13	KC861529
Phymatolithon calcareum	Le Dragon, Glénan, Brittany, -15 m, 13/05/2011. Coll: T. Wilfried, Y. Fontana.	CPVP-916	MAERL039-11	KC861589
Phymatolithon calcareum	Ensenada de Bornalle, Ría de Muros-Noia, Galicia, -11 m, 31/03/2011. Coll: I. Bárbara, C. Pardo, R. Barreiro, V. Peña.	CPVP-560	MAERL042-11	KC861592
Phymatolithon calcareum	Nido do Corvo, Ría de Arousa, Galicia, -6 m, 23/11/2010. Coll: I. Bárbara, C. Pardo, R. Barreiro, V. Peña.	CPVP-164	MAERL115-13	KC861567
Phymatolithon calcareum	Punta Barbafeita, Ría de Arousa, Galicia, -5 m, 23/06/2011. Coll: I. Bárbara, F. Bunker, V. Peña.	CPVP-1067	MAERL101-13	KC861581
Phymatolithon calcareum	Isla Benencia, Ría de Arousa, Galicia, -3 m, 23/06/2011. Coll: I. Bárbara, F. Bunker, V. Peña.	CPVP-1108	MAERL110-13	KC861572
Phymatolithon calcareum	Isla de Ons, Ría de Pontevedra, Galicia, -13 m, 07/04/2011. Coll: I. Bárbara, R. Barreiro, V. Peña.	CPVP-607	MAERL131-13	KC861551
Phymatolithon calcareum	Playa de Tulla, Ría de Pontevedra, Galicia, -9 m, 07/04/2011. Coll: I. Bárbara, R. Barreiro, V. Peña.	CPVP-628	MAERL036-11	KC861586
Phymatolithon calcareum	Islas Cíes, Ría de Vigo, Galicia, -11 m, 03/04/2011. Coll: I. Bárbara, R. Barreiro, V. Peña.	CPVP-655	MAERL113-13	KC861569
Phymatolithon calcareum	Baliza Tofiño, Ría de Vigo, Galicia, -11 m, 03/04/2011. Coll: I. Bárbara, R. Barreiro, V. Peña.	CPVP-753	MAERL136-13	KC861546

Uncultured Corallinales	Atlantic France, Bittner *et al.* (2010).	LBC0001	-	GQ917247
Lithothamnion corallioides (P.L.Crouan & H.M.Crouan) P.L.Crouan & H.M.Crouan	Baie de Douarnenez, Finistère, Brittany, -10 m, 08/03/2011. Coll: J. Grall.	CPVP-802	MAERL017-11	KC861447
Lithothamnion glaciale Kjellman	English Harbour Eastern Cove, Newfoundland and Labrador. *Unpublished.*	GWS007542 (ABMMC9561-10)	GWS007542	HM918812
Mesophyllum sphaericum V. Peña, Bárbara, W.H. Adey, Riosmena-Rodríguez & H.G. Choi	Isla Benencia, Ría de Arousa, Galicia, -3 m, 14/10/2008. Coll: I. Bárbara, V. Peña. Holotype material.	CPVP-776 (SANT-Algae 21804)	MAERL015-11	KC861526
Mesophyllum erubescens (Foslie) Me. Lemoine	Hawaii, Pacific Ocean, Sherwood *et al.* (2010a).	ARS02835	-	HQ422717

P.L. Crouan & H.M. Crouan and *Mesophyllum sphaericum* V. Peña, Bárbara, W.H. Adey, Riosmena-Rodríguez & H.G. Choi, the latter were produced from the holotype material (Peña *et al.* 2011, **Table 1**). DNA barcodes of *P. calcareum* were compared to public records in Genbank and BOLD databases. Available COI-5P sequences from Genbank and BOLD for the subfamily Melobesioideae were included in the analyses as outgroup (**Table 1**). Distance analyses for the total of 24 COI-5P sequences were performed using Neighbor-Joining (N-J) algorithm (Jukes-Cantor method) with default settings in Mega v.6.0 (Tamura *et al.* 2013).

RESULTS

The DNA barcodes produced for these gametophytes were 100% identical to COI-5P sequences obtained from neotype material for *Phymatolithon calcareum* (possessing multiporate sporangial conceptacles, Woelkerling & Irvine 1986) and from recent collections of this taxon along Atlantic European maerl beds (**Fig. 2**). In the N-J tree, *P. calcareum* clearly

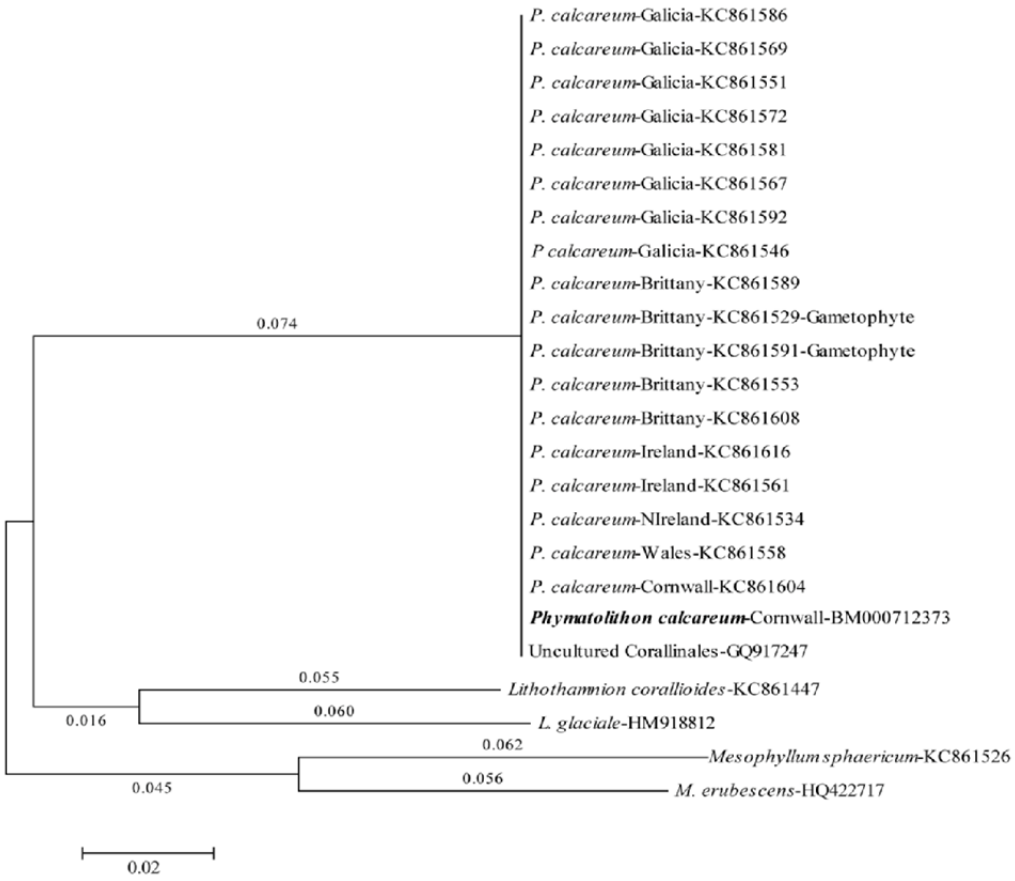


Fig. 2. Neighbor-Joining tree for DNA barcodes (COI-5P) of *Phymatolithon calcareum* (detailed in **Table 1**). The outgroup contained other members of the subfamily Melobesioideae from our on-going project MAERL and from GenBank: *Lithothamnion* (*L. corallioides*, *L. glaciale*), and *Mesophyllum* (*M. sphaericum*, *M. erubescens*). Evolutionary distances of the branches were computed using the Jukes-Cantor method. Scale bar refers to base subtitutions per site.

separated from other Melobesioideae (*Lithothamnion* and *Mesophyllum*). Genbank and BOLD searches reveraled that only one accession matched our DNA barcodes for *P. calcareum*; the matching sequence was produced by a collection from the Atlantic coast of France labeled as *Uncultured Corallinales* (accession no. GQ917247, Bittner *et al.* 2010, **Table 1**).

The gametophytes of *P. calcareum* were encrusting thalli with short branches, up to 5 cm of diameter, growing on pebbles associated to the maerl bed (**Fig. 3a,b**). The uniporate conceptacles examined here were raised, appearing as white circles scattered on the thallus surface (**Fig. 3c,d**). The anatomical examination by SEM revealed branches with abundant uniporate conceptacles raised, conical to hemispherical, 250-300 µm external diameter in surface view, with a pore of 15-17 µm of diameter (**Fig. 4a,b,c**). In transverse section (**Fig. 4d**), the uniporate conceptacles were empty and were located within the cortex. The conceptacle chambers were elliptical-ovoid, 226-260 µm diameter by 85-140 µm height. The pore canal

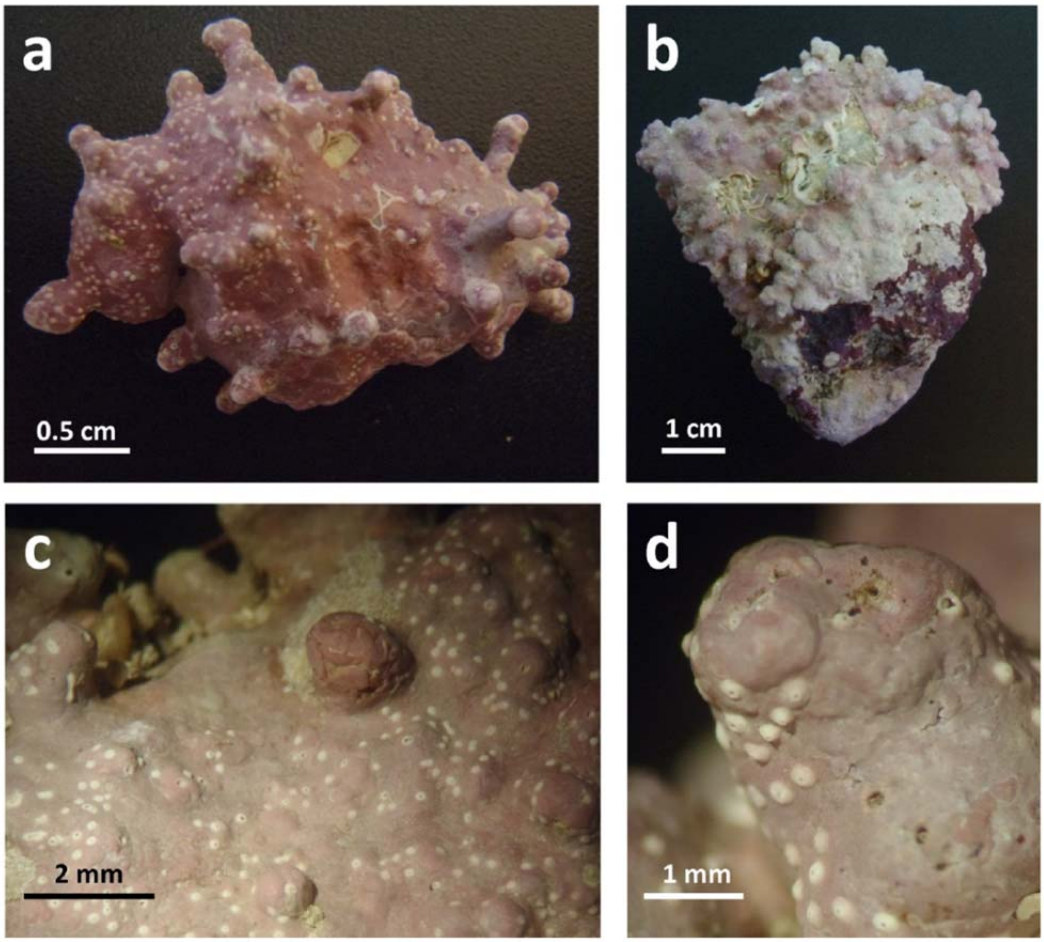


Fig. 3. Gametangial plants of *Phymatolithon calcareum*. **a-b.** Encrusting branched plants growing on pebbles (CPVP-955 and CPVP-943, respectively). **c.** Uniporate female conceptacles scattered along the thallus surface. **d.** Uniporate female conceptacles raised, on branches.

was 130 µm long, tapering from 83 µm at the base to 23 µm wide at the surface, with lining filaments oriented to the canal. Although we only found empty chambers, they were interpreted as mature female conceptacles given their shape and large size. The epithallial cells were domed, the subepithallial cells were short and cells fusions were abundant between adjacent cortical cells (**Fig. 4e**). The epithallial cells surface was *Phymatolithon*-type with thickened calcareous ridges surrounding central concavities (**Fig. 4f**).

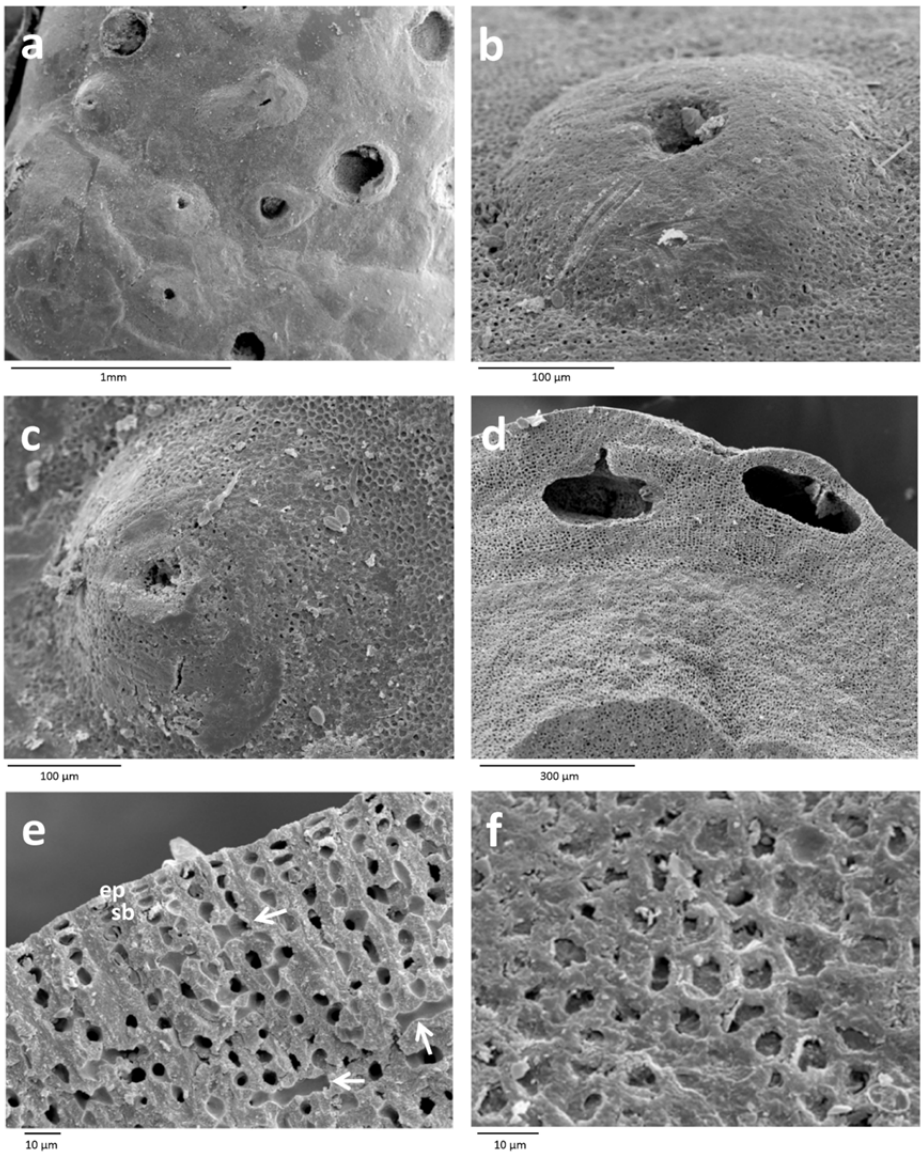


Fig. 4. Uniporate female conceptacles of *Phymatolithon calcareum* and vegetative diagnostic features (SEM images). **a.** Surface of a branch with abundant uniporate conceptacles. **b-c**. Surface view of uniporate female conceptacles raised, hemispherical to conical. **d.** Vertical section of the thallus with empty uniporate female conceptacles showing chambers elliptical to ovoid with a canal pore. **e.** Vertical section of the thallus showing domed epithallial cells (ep), short subepithallial initials (sb), and cell fusions between contiguous cortical cells (arrows). **f.** Surface view of the thallus showing epithallial cells *Phymatolithon*-like with thickened calcareous ridges surrounding central concavities.

DISCUSSION

Our study illustrates the usefulness and accuracy of DNA barcodes for assessing the diversity of maerl-forming species. In agreement with the literature, our results indicate that *Phymatolithon calcareum* is a common component of maerl beds in Brittany, Ireland, Britain and Galicia (Lemoine 1910, Cabioch 1969, Adey & McKibbin 1970, Bosence 1976, Irvine & Chamberlain 1994, Peña & Bárbara 2004, Peña & Bárbara 2008b). Our molecular results, which include the neotype of *P. calcareum* (Woelkerling & Irvine 1986), confirm that the encrusting, epilithic plants found in an Atlantic European bed were gametophytes of *P. calcareum*. The anatomical examination of the gametophytes showed raised conceptacles with empty chambers that were interpreted as mature female conceptacles, although previous authors described the uniporate female conceptacles as not prominent, with only the canal pore slightly protruding and smaller dimensions (120-150 µm of diameter by 60-110 µm in height with a canal pore 50 µm long; Mendoza & Cabioch 1998, Bressan & Babbini 2003). However, the vegetative features observed in our specimens matched the diagnostic characters described for *P. calcareum* in the literature (Cabioch 1966, Adey & McKibbin 1970, Chamberlain 1990, Irvine & Chamberlain 1994).

A previous study carried out also in the same Atlantic region (Brittany) reported uniporate conceptacles only in crustose plants attached to gravel or dead maerl (Mendoza & Cabioch 1998). Interestingly, however, gametangial plants have been reported to grow as maerl in the Mediterranean and Alaska (Bressan & Babbini 2003, Konar *et al.* 2006). A previous study in European waters detected fertile sexual plants of *P. calcareum* in September-October (Mendoza & Cabioch 1998) while our specimens were collected in May. On the other hand, Konar *et al.* (2006) found male gametangial plants in summer at much higher latitude (Alaska). In this regard, the fact that our conceptacles were empty might indicate that they had released their content some time before collection. However, the conceptacles were abundant along the surface of the plant rather than buried within the thallus.

Based on our collections of more than 1000 unattached and encrusting plants from a range of Atlantic European maerl beds sampled at different times of the year (Carro *et al.* 2014, Pardo *et al.* 2014a), the occurrence of uniporate sexual conceptacles in *P. calcareum* seems a rather infrequent phenomenon. In Britain, gametangial plants of *P. calcareum* remain unreported and it is the only species of this genus with unrecorded sexual structures in this region (Irvine & Chamberlain 1994). In comparison, multiporate sporangial conceptacles were

commonly observed on unattached plants throughout the year and they even occur in the neotype material (Lemoine 1910, Suneson 1958, Cabioch 1969, Adey & McKibbin 1970, Woelkerling & Irvine 1986, Irvine & Chamberlain 1994, Peña & Bárbara 2004, 2008b). The extremely low frequency of uniporate sexual conceptacles could be indicative that the gametophytes spend most of their life history as vegetative plants. Alternatively, their scarcity could be just a consequence of a low ratio of gametophytes in Atlantic European beds. Cabioch (1969) suggested that the rarity of sexual and asexual reproduction in Breton maerl beds possibly indicated that reproduction follows a cyclical pattern of at least 6 years with alternate fertile periods between the main maerl-forming species *P. calcareum* and *Lithothamnion corallioides*. On the other hand, the low occurrence of fertile plants in Brittany and Norway was interpreted as evidence that recruitment in maerl populations depends primarily on the breakage of branches of attached plants (Cabioch 1969, Freiwald 1995). However, given the absence, or at least scarcity, of encrusting thalli of *P. calcareum* reported so far, it seems unlikely that they might be the providers of unattached plants in most of the Atlantic European beds. According to Irvine & Chamberlain (1994), encrusting *P. calcareum* has not ever been recorded in Britain. Likewise, other than the collections from Breton maerl beds reported here, we never detected encrusting *P. calcareum* in any other Atlantic European bed. In this regard, our results seem consistent with the proposal that thallus fragmentation of the maerl itself must be the main mechanism of propagation in Atlantic beds as previously suggested for unattached populations in the literature (Bosence 1976, Johansen 1981, Steller & Foster 1995). However, solving the precise recruitment strategy followed by Atlantic maerl populations surely warrants more detailed investigations.

CHAPTER 4

Development and multiplexing of the first microsatellite markers in a coralline red alga (*Phymatolithon calcareum*, Rhodophyta)

This chapter is based on content published in:

Pardo C., Peña V., Bárbara I., Valero M. & Barreiro R. (2014). Development and multiplexing of the first microsatellite markers in a coralline red alga (Phymatolithon calcareum, Rhodophyta). Phycologia 53, 474-479.

DEVELOPMENT AND MULTIPLEXING OF THE FIRST MICROSATELLITE MARKERS IN A CORALLINE RED ALGA (*PHYMATOLITHON CALCAREUM*, RHODOPHYTA)

ABSTRACT

Coralline red algae are important habitat builders in coastal waters around the world but their population genetics has been largely overlooked because of a lack of appropriate markers. Here, next generation sequencing was used to identify the first set of microsatellite loci ever developed for a coralline alga, *Phymatolithon calcareum*, a common builder of maerl beds, particularly in Europe. Eleven polymorphic microsatellite markers were identified, of which eight were particularly suitable for population and individual-based applications. In these, the number of alleles ranged from two to five, while expected heterozygosities varied from 0.143 to 0.803. Two multiplex polymerase chain reactions were designed and proved to successfully amplify these loci in specimens collected along the European Atlantic. The new set of markers will enable the study of population genetic diversity and connectivity patterns of these non-geniculate corallines that are especially relevant to propose conservation actions.

KEYWORDS: conservation, Hapalidiales, Europe, maerl, microsatellites, multiplexed PCR, NGS, seaweed, SSR, 454.

INTRODUCTION

Corallines are calcified red algae with a worldwide distribution. These algae play a critical ecological role because they build and consolidate habitats that accommodate a high biodiversity (Steneck 1986, Foster 2001, Nelson 2009). In particular, the accumulations of free-living, non-geniculate coralline algae known as maerl beds are one of the Earth's ''Big Four'' benthic communities dominated by marine macrophytes (Foster 2001). These beds harbour broodstock bivalves and serve as nursery areas for the juveniles of commercially valuable fish, crabs, and scallops (Kamenos *et al.* 2004a-d). However, a number of studies reveal that maerl beds have declined in both extent and quality along the European coasts due to anthropogenic pressure (reviewed in Hall-Spencer *et al.* 2010). Despite their relevance, corallines in general, and maerl-forming species in particular, have been largely excluded from DNA fingerprinting and population genetics studies because of a shortage of appropriate markers (exceptions are Pearson & Murray 1997, and Schaeffer *et al.* 2002). As a result, we lack important information for their management and conservation, such as genetic diversity, connectivity among populations, or recruitment levels (Bosence 1976, Foster 2001).

Nuclear microsatellites, also called simple sequence repeats (SSRs), are short tandem repeats of 1–6 nucleotide motifs that are assumed to be randomly distributed throughout the genome (Jarne & Lagoda 1996, Goldstein & Schlotterer 1999). Their high mutation rate, simple inheritance, and codominance make them appropriate markers for describing patterns of intraspecific genetic diversity in applications that range from ancient DNA studies, breeding pedigree analyses, population genetics, or conservation/management (Jarne & Lagoda 1996, Goldstein & Schlotterer 1999, Zane *et al.* 2002). In the particular case of red seaweeds, microsatellites have been used to assess fertilization success and male gamete dispersal distance by paternity analyses (Engel *et al.* 2004), to detect patterns of gene flow and mating systems at microgeographic scale in the intertidal landscape (Engel *et al.* 2004, Krueger-Hadfield *et al.* 2013), to evaluate connectivity and genetic differentiation at large geographic scale (Andreakis *et al.* 2009, Hu *et al.* 2011, Kostamo *et al.* 2012, Provan *et al.* 2013), to assess the genetic effects of domestication by comparing wild and cultivated populations (Guillemin *et al.* 2008), and for the construction of genetic linkage maps for breeding studies (Chaotian *et al.* 2010).

Here, we have developed microsatellite markers for *Phymatolithon calcareum* (Pallas) W.H. Adey & D.L. McKibbin, one of the two maerl-forming corallines currently listed in Annex V

of the European Union Habitats Directive as a species of community interest. Commonly considered a major component of maerl beds in Europe (Hall-Spencer *et al.* 2010), the widespread distribution of *P. calcareum* from the British Isles to the Iberian Peninsula was recently confirmed with DNA barcode data (Carro *et al.* 2014). Several aspects of its biology and ecology have received attention in the past (e.g. Cabioch 1966, Adey & McKibbin 1970, Cabioch 1970, Wilson *et al.* 2004), but no study has ever addressed its population genetics so far. As a result, we ignore the potential of the species to face future environmental changes: whether the beds in a region should be treated as a single panmictic unit or as differentiated entities, the haploid/diploid ratio of individuals, or the significance of asexual reproduction (Peña *et al.* 2014b). To our knowledge, this is the first time that microsatellite loci are developed for a coralline. In fact, microsatellite development has received little attention in seaweeds in general and in Rhodophyta in particular (Andreakis *et al.* 2007). A review of the literature reveals that polymorphic, validated microsatellite markers have been reported for only 19 species of red algae from six orders (**Table S1**). Most efforts have focused on commercially valuable seaweeds (14 species) while nonnative organisms (*Asparagopsis taxiformis*, *Acanthophora spicifera, Gracilaria vermiculophylla*) or those of conservation concern (*Pseudopolyides furcellarioides*, *Grateloupia lanceola*) have received little attention (see **Table S1** for references). In our case, the microsatellite loci were detected with next generation sequencing (NGS) technology given the successful application of this technology to other non-model and not previously sequenced organisms (Castoe *et al.* 2010, Takayama *et al.* 2011, Kale *et al.* 2012, López-Uribe *et al.* 2013). Specifically, NGS was used for the first time by Couceiro *et al.* (2011a) to develop microsatellite markers for Rhodophyta. Moreover, genotyping throughput and cost effectiveness were improved by incorporating the set of polymorphic microsatellite loci developed in this study into a multiplexed Polymerase Chain Reaction (PCR) design.

MATEIRAL AND METHODS

Genomic DNA was extracted and purified from individuals of *Phymatolithon calcareum* using the DNeasy® Blood & Tissue Kit (Qiagen Inc., Valencia, California USA) following the manufacturer's recommendations with minor modifications. As the branched morphology and rugged surface of maerl favours the growth of epiphytes, we followed a number of steps to ensure that the extracted DNA was uncontaminated with that of other species. Before extraction, each individual was carefully cleaned from epiphytes under the stereomicroscope

with the help of an electric miniature drill brush. DNA extractions were restricted to the outer living layer obtained by carefully grinding the surface of each individual with an electric drill bit. The taxonomic identity of each DNA sample was confirmed with DNA barcodes by comparison with records MAERL011-11 to MAERL097-11 and MAERL098-13 to MAERL237-13 filed in Barcode of Life Data Systems (BOLD, http://www.boldsystems.org, project ''maerl-NE Atlantic'', code MAERL). Total genomic DNA was sent to GenoScreen (Lille, France). Equimolar amounts of DNA from 11 individuals were combined to yield a total of 1 µg that was used for the development of microsatellite-enriched libraries through 454 GS-FLX Titanium pyrosequencing, as described in Malausa *et al.* (2011). Briefly, total DNA was enriched for AG, AC, AAC, AAG, AGG, ACG, ACAT, and ATCT repeat motifs and subsequently amplified. After purification and quantification, PCR products were used to construct GS-FLX libraries following the manufacturer's protocols (Roche Diagnostics Corporation, Branford, Connecticut USA) that were sequenced on a GS-FLX-PicoTiterPlate device. After filtering for redundancy with the program QDD (Meglécz *et al.* 2010), sequences were searched for microsatellite inserts with perfect motifs. Then, the version of Primer3 implemented in QDD was run to design primer pairs for each sequence (Rozen & Skaletsky 2000, http://primer3. sourceforge.net/), and these were tested for amplification (i.e. validated) on seven DNA samples. A subset of the validated primer pairs was tested for polymorphism on 21 specimens from 16 locations covering three regions along the Atlantic Ocean range of the species: the British Isles, the Atlantic coast of France, and the Atlantic coast of Spain (for more details see **Table S2**). Initially, 48 primer pairs were tested for amplification and polymorphism with six individuals from the British Isles (BI), five from the Atlantic coast of France (AF), and 10 from the Atlantic coast of Spain (AS). Owing to the low number of polymorphic loci detected in these initial trials, amplification and polymorphism were tested in another 63 primer pairs using six individuals from BI, seven from AF, and eight from AS.

PCR amplifications were performed in 25 µL reactions containing 20 ng of template DNA, 1X reaction buffer, 37.5 pmol $MgCl_2$, 6 pmol dNTPs, 10 pmol of labelled primer, 1 pmol of unlabeled primer, and 1 U Taq DNA polymerase (FastStart, Roche Diagnostics GmbH, Mannheim, Germany). PCR cycling consisted of an initial denaturation at 95ºC for 10 min, followed by 40 cycles: denaturation at 95ºC for 30 s, annealing at 55ºC for 30 s, and extension at 72ºC for 1 min, followed by a final extension at 72ºC for 10 min. Each microsatellite amplification was diluted with dH_2O (1:50), mixed with Hi-Di™ Formamide and GeneScan™ 500 LIZ® Size Standard (Applied Biosystems, Foster City, California, USA). Fragments were

separated using an Applied Biosystems 3730xl DNA Analyzer. Alleles were scored using GeneMapper® v.4.0 (Applied Biosystems). Polymorphism was assessed as the number of alleles per locus and per region, the observed and expected heterozygosities (H_O and H_E), and the Polymorphism Information Content (PIC) index were calculated with The Excel Microsatellite Toolkit (Park 2001).

Multiplex PCR reactions were designed with the help of the Multiplex Manager software (Holleley & Geerts 2009). Multiplex performance was tested in 20 µL reactions containing 1 µL of template DNA, 10 µL of Qiagen® Multiplex PCR Master Mix (Qiagen), and 2 µL of primer mix. The primer mix contained 2 µM of each primer except primer pair PC-8 (4 µM). PCR cycling consisted of an initial denaturing at 95ºC for 15 min, followed by 25 cycles of denaturation at 94ºC for 30 s, annealing at 59ºC for 90 s, and extension at 72ºC for 60 s; followed by a final extension at 60ºC for 30 min. Multiplexed PCR products were separated on an Applied Biosystems 3730XL DNA analyzer at Macrogen Inc. (Seoul, Korea). Electropherograms were analysed and scored with the help of GeneMarker® v.1.7.0 software (SoftGenetics, State College, Pennsylvania USA).

RESULTS

Of the 17,539 reads obtained (average length: 240.4 bp), 2036 (11.6%) contained microsatellite inserts with perfect motifs. Of these, 111 were suitable for primer design with expected amplicon sizes ranging from 90 bp to 285 bp. Most of these 111 motifs were tri- (47.7%) and dinucleotides (36.9%), while larger ones were rare (10.8% tetra-, 3.6% hexa-, and 0.9% pentanucleotides). Of the 111 potentially amplificable loci, 42 primer pairs were biologically validated, i.e. produced specific PCR products of the expected size, and 24 of them were tested for polymorphism. Eleven of these 24 loci (45.8%) turned out to be polymorphic under the criterion that they produced a minimum of two distinct genotypes (**Table 1**). Unlike the initial set of potential amplicons, the 11 polymorphic loci were dominated by tetranucleotide motifs (54.5%) followed by di- (27.3%) and trinucleotides (18.2%).

Observed heterozygosities varied from 0.0 to 1.0, while expected heterozygosities ranged from 0.000 to 0.803 (**Table 2**). The average number of alleles per locus was 2.9 (range: 2– 5): three loci were moderately polymorphic (4–5 alleles) and eight had low polymorphism (2–3 alleles). Among the latter, two loci that produced a single allele at several populations (PC-10 and PC-11) were considered of little use for future population studies, and they were excluded from the multiplex design. Locus PC-9 was also excluded from the multiplex reactions

because it failed to amplify in four out of the 21 DNA samples. Locus PC-8 was polymorphic (H_E varied from 0.44 to 0.71) but the absence of heterozygotes suggests that this locus is either haploid (located in the mitochondrial or plastidial genome) or diploid but characterized by a high frequency of null alleles. The latter seems more likely if we consider that PC-8 also failed to amplify in 2 of the 21 individuals. Consequently, PC-8 was temporarily retained for the multiplex design but its results must be interpreted with caution to confirm/refute the occurrence of null alleles with further data. The eight retained loci were successfully combined in two multiplexed reactions (**Table 1**) that yielded clear profiles, matched the peaks observed in single-locus PCRs, and were straightforward to score. PIC values per population in these eight loci ranged from 0.124 to 0.687 with a mean of 0.434. Following Bostein *et al.* (1980), two loci (PC-1 and PC-3) with mean PIC across populations > 0.5 can be regarded as highly informative, while five are reasonably informative (mean PIC between 0.25 and 0.5), and one (PC-4) is slightly informative (mean PIC , 0.25).

Table 1. Primers for 11 polymorphic microsatellite loci in *Phymatolithon calcareum* and multiplexed PCR design.

Locus	GenBank accesion no.	Primer sequence (5'-3')	Repeat motif	Size range (bp)	Multiplex PCR	Concentration (µM)	Primer Fluorescent tag
PC-1	KF768765	F: ATTTATTTGGCCCAACTCGT	$(CA)_{11}$	130-144	1	2 µM	PET
		R: CGCTTGTGTCCTTTTCGTCT			1	2 µM	
PC-2	KF768768	F: CCGTCCATCCAACACGAA	$(GAG)_7$	132-138	1	2 µM	VIC
		R: CATCAATCCGTAACATGAGCA			1	2 µM	
PC-3	KF768770	F: CACTCGTTGATCATGGCAC	$(CACT)_{17}$	258-297	1	2 µM	6-FAM
		R: TTACAACGAGGTTCGAAAGG			1	2 µM	
PC-4	KF768771	F: GATTGCGAGTCAAATGCTGA	$(GGAT)_5$	97-101	1	2 µM	NED
		R: TGCAATGATAATTGACTAAGCGA			1	2 µM	
PC-5	KF768774	F: CAGGAGCAATTTCACAGCAG	$(TCCA)_6$	162-169	1	2 µM	6-FAM
		R: GGAGGATCAGATGAAGGGGT			1	2 µM	
PC-6	KF768764	F: GGGAACGGATTGTTATTGGA	$(AC)_8$	101-103	2	2 µM	NED
		R: CCGTTTTCGGTAATCATGTTG			2	2 µM	
PC-7	KF768767	F: CCCTTCAACTTTGCCAATTC	$(CAT)_9$	102-119	2	2 µM	VIC
		R: CAGTTTTGGATAAGGCGGAA			2	2 µM	
PC-8	KF768766	F: TAAACGAAAGAGAATGAAAGTAAAGC	$(AC)_6$	151-161	2	4 µM	6-FAM
		R: GGTCTCCCCATTCTGGCT			2	4 µM	
PC-9	KF768772	F: TGGTGGTTTTATTCTTCGCA	$(CATT)_5$	88-104			
		R: TGCTAGACCCATTACGGGAA					
PC-10	KF768769	F: CATCAGCTGAACCCACTTCA	$(ATCC)_8$	217-221			
		R: TGCCAATCAAAGTGAGGAGA					
PC-11	KF768773	F: CTGACCTTGGCTGTGCTGT	$(GATG)_7$	133-142			
		R: CGGGAGTCATTCCAGACATT					

Table 2. Characteristics of 11 polymorphic microsatellite loci in *Phymatolithon calcareum* collections sampled in three regions along its European Atlantic range (BI, Brithish Isles; AF, Atlantic France; AS, Atlantic Spain). Loci selected for multiplex reactions are indicated in bold. *n*, sample size as number of successful DNA amplifications; N_I, number of DNA samples (individuals) assayed; N_A, number of observed alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; PIC, polyporphism information content.

Locus	Region	n/N_I	N_A	H_O	H_E	PIC
PC-1	BI	6/6	4	0.833	0.758	0.639
	AF	5/6	2	0.200	0.467	0.332
	AS	10/10	3	0.800	0.700	0.591
	Total	21/21	4			
PC-2	BI	6/6	2	0.333	0.303	0.239
	AF	7/7	2	0.571	0.440	0.325
	AS	8/8	3	0.500	0.425	0.354
	Total	21/21	3			
PC-3	BI	6/6	4	0.833	0.803	0.687
	AF	5/5	4	0.600	0.778	0.645
	AS	10/10	4	1.000	0.753	0.665
	Total	21/21	4			
PC-4	BI	6/6	2	0.167	0.167	0.141
	AF	7/7	2	0.143	0.143	0.124
	AS	8/8	2	0.375	0.525	0.371
	Total	21/21	2			
PC-5	BI	6/6	3	0.667	0.682	0.555
	AF	7/7	3	0.429	0.670	0.551
	AS	8/8	3	0.375	0.342	0.294
	Total	21/21	3			
PC-6	BI	6/6	2	0.500	0.530	0.368
	AF	5/5	2	0.400	0.533	0.365
	AS	10/10	2	0.100	0.395	0.305
	Total	21/21	2			
PC-7	BI	6/6	3	0.667	0.712	0.579
	AF	7/7	3	0.571	0.604	0.465
	AS	8/8	4	0.625	0.517	0.443
	Total	21/21	5			
PC-8	BI	5/6	3	0.000	0.711	0.563
	AF	7/7	3	0.000	0.615	0.501
	AS	7/8	2	0.000	0.440	0.325
	Total	19/21	3			
PC-9	BI	5/6	2	0.800	0.533	0.365
	AF	6/7	2	0.833	0.530	0.368
	AS	6/8	2	0.833	0.530	0.368
	Total	17/21	2			
PC-10	BI	6/6	2	0.167	0.167	0.141
	AF	5/5	1	0.000	0.000	0.000
	AS	10/10	1	0.000	0.000	0.000
	Total	21/21	2			
PC-11	BI	6/6	1	0.000	0.000	0.000
	AF	7/7	2	0.143	0.143	0.124
	AS	8/8	1	0.000	0.000	0.000
	Total	21/21	2			

DISCUSSION

The availability of new markers with high resolving power will likely help to settle several features of maerl that remain poorly known due to the lack of appropriate data. For example, maerl beds of *Phymatolithon calcareum* in the European Atlantic Ocean are thought to contain only tetrasporophytes, since the sexual gametophyte has rarely been detected (Peña *et al.* 2014b). This assumption is almost impossible to test with conventional, morphology-based approaches because non-reproductive specimens overwhelmingly dominate these beds. In contrast, haploid gametophytes should be readily distinguished from diploid tetrasporophytes using a combination of codominant microsatellite markers using heterozygocity as a signature of diploidy. In this regard, the multilocus genotype of the 21 specimens used in this study revealed that each one of them included at least one heterozygous locus indicating that they were all diploid tetrasporophytes. It could be argued that the unnoticed occurrence of fertilized gametophytes (i.e. specimens that might combine haploid and diploid structures) could lead to misclassify haploid females as tetrasporophytes. However, (i) the dominance of non-reproductive specimens together with (ii) the fact that we never observed more than two alleles per locus rule out the possibility that DNA from the zygotes was amplified.

Another unresolved issue where microsatellites can be helpful is in elucidating the mechanisms that sustain maerl beds. The lack of gametophytes led to the speculation that vegetative fragmentation should be a major source of new recruits in these beds (Bosence 1976, Johansen 1981). If so, beds should contain a sizeable portion of clone mates sharing the same multilocus genotype. This prediction can be tested with microsatellite loci by examining the number of identical multilocus genotypes. In our data set, *G/N* ratios (where *G* = no. of genotypes and *N* = no. of individuals) were 0.875 in BI, 0.923 in AS, and 1 in AF, indicating that most individuals did not share the same genotype. Further studies with larger sample sizes are needed to explore this question in more detail. Nevertheless, the set of markers developed here seems to show enough resolving power to address this issue.

Most seaweeds are thought to disperse mostly over short distances with occasional episodes of long-range dispersal (see Gaylord *et al.* 2002 for references) but the actual pattern of genetic connectivity between maerl beds has never been measured (Hall-Spencer *et al.* 2010). Again, our set of microsatellites should provide estimates about the extent of propagule exchange among local populations. Finally, our set of microsatellite markers may also serve to assess the conservation value of particular maerl beds. To our knowledge, this approach was

previously applied to other red algae using other molecular markers such as Amplified Fragment Length Polymorphisms (AFLPs; e.g. Couceiro *et al.* 2011b) but there is no reason to suspect that microsatellites may not serve the same purpose.

The number of polymorphic loci detected in this study resembles the values typically observed in other red algae if we exclude a few commercial species that have undergone larger development efforts (see **Table S1** and references therein). Also, the number of alleles per locus in *Phymatolithon calcareum* was similar to the values reported for other red algae (**Table S1**), indicating that the low–moderate polymorphism detected in *P. calcareum* may be typical of many Rhodophyta (see also Andreakis *et al.* 2007). While our initial set of potential amplicons was clearly dominated by trinucleotides, our final set of polymorphic loci was dominated by tetranucleotide motifs suggesting that future attempts to identify polymorphic loci in these red algae might see their efficiency increased by an enrichment step more focused on this motif size. The predominance of trinucleotides in our potential amplicons was likely due to the enrichment step used in this study, as previous NGS estimates of microsatellite density in unenriched DNA indicate that dinucleotide repeats can be overwhelmingly dominant (> 83%) in Rhodophyta (Couceiro *et al.* 2011a).

In summary, using NGS technology we have developed the first set of polymorphic loci for a coralline red algae. Eight microsatellite loci that seem promising for future population genetics studies in the maerl-forming *Phymatolithon calcareum* were successfully incorporated into a multiplexed PCR design to facilitate genotyping throughput. Our results indicate that the multilocus genotypes obtained with this set of markers should shed light on a number of pending issues about the biology of maerl: the genetic diversity of local populations, the pattern of connectivity at various spatial scales (local, regional), the mating system, and the haploid/diploid ratios of natural populations. Altogether, this information will allow us to design management actions by identifying populations/beds of high conservation value (high within-population genetic diversity and/or distinctive genetic composition) (Frankham *et al.* 2004).

SUPPLEMENTAL DATA

Table S1. Characteristics of the polymorphic microsatellite loci developed for Rhodophyta algae. Only studies that reported validated loci are included. P = perfect motif, IM = imperfect, I = interrupted, C = composite, N.A. = information not available.

Order	Species	No. (and type) of loci / allele no. / diversity (as H_E unless otherwise stated)	Reference
Bangiales	*Porphyra lines:*	25 (23P, 2I) / 2-9/ N.A.	Sun *et al.* (2006)
	- *Porphyra yezoensis* Ueda (currently *Pyropia yezoensis* (Ueda) M.S. Hwang & H.G. Choi) - *Porphyra haitanensis* T.J. Chang & B.F. Zheng (currently *Pyropia haitanensis* (T.J. Chang & B.F. Zheng) N. Kikuchi & M. Miyata) - *Porphyra oligospermatangia* C.K. Tseng & B.F. Zheng - *Porphyra katadae* A. Miura (currently *Pyropia katadae* (A. Miur) M.S. Hwang, H.G. Choi, N. Kikuch & M. Miyata)		
	Porphyra haitanensis	11 (7P, 4I) / 3-6 / 0.182-0.955 (H_O)	Zuo *et al.* (2007)
	Porphyra haitanensis	28 (27P, 1I) / 4-15/ 0.49-0.75	Xie *et al.* (2009)
	Porphyra haitanensis	37 (P) / 2-4/ 0.206-0.289	Development by Hu *et al.* (2006). Validation by Bi *et al.* (2014)
	Porphyra yezoensis	13 (P) / N.A. / N.A.	Liu *et al.* (2005)
	Porphyra yezoensis	12 (2P, 9I, 1C) / 2-4 / 0.03-0.65	Kong *et al.* (2009)
Bonnemaisoniales	*Asparagopsis taxiformis* (Delile) Trevisan de Saint-Léon	8 (1P, 7I) / 2-7 / 0.83-0.91	Andreakis *et al.* (2007)
Ceramiales	*Acanthophora spicifera* (M. Vahl) Børgesen	5 (I) / 6-13 / 0.030-0.278 (Shannon Index)	O'Doherty & Sherwood (2007)
Gigartinales	*Chondrus crispus* Stackhouse	10 (3P, 7I) / 3-46 / 0.253-0.939	Krueger-Hadfield *et al.* (2011)
	Chondrus crispus	8 (P) / 2-12 / 0.105-0.436	Provan & Maggs (2012)
	Furcellaria lumbricalis (Hudson) J.V. Lamouroux	10 (5P, 4IM, 1I) / 8-17 (neutral loci), 4-12 (EST-derived) / 0.614-0.789 (neutral loci), 0.217-0.420 (EST-derived)	Kostamo *et al.* (2012)
	Pseudopolyides furcellarioides Gallardo, Bárbara & Cremades	5 (P) / 3-4 / 0.254-0.656	Couceiro *et al.* (2011a)

Gracilariales	*Gracilaria birdiae* Plastino & E.C. Oliveira	13 (6P, 1I, 6IM) / 2-15 / 0.059-0.854	Ayres-Ostrock *et al.* (2015)
	Gracilaria caudate J. Agardh	16 (11P, 2IM, 3I) / 2-10 / 0.059-0.810	Ayres-Ostrock *et al.* (2015)
	Gracilaria chilensis C.J. Bird, McLachlan & E.C. Oliveira	6 (1P, 4I, 1C) / 2-3 / 0.000-0.511	Guillemin *et al.* (2005)
	Gracilaria gracilis (Stackhouse) M. Steentoft, L.M. Irvine & W.F. Farnham	2 (1P, 1IM) / 10-22 / N.A.	Wattier *et al.* (1997)
	Gracilaria gracilis	9 (3P, 5I, 1C) / 2-7 / 0.000-0.640	Luo *et al.* (1999)
	Gracilaria species: - *Gracilaria changii* (B.M. Xia & I.A. Abbott) I.A. Abbott, J. Zhang & B.M. Xia - *Gracilaria gracilis*. - *Gracilariopsis lemaneiformis* (Bory de Saint-Vincent) E.Y. Dawson, Acleto & Foldvik	1 (P) / 5 / N.A.	Song *et al.* (2013)
	Gracilaria tenuistipitata C.F. Chang & B.M. Xia	2 (P)/ N.A. / N.A.	Song *et al.* (2014)
	Gracilaria vermiculophylla (Omhi) Papenfuss	9 (P)/ 1-5 / 0.063-0.682	Kollars *et al.* (2015)
Halymeniales	*Grateloupia lanceola* (J. Agardh) J. Agardh	7 (P) / 2-4 / 0.034-0.531	Couceiro *et al.* (2011a)
	Grateloupia filicina (J.V. Lamouroux) C. Agardh	40 (34P, 5I, 1C) / 3-10 / 0.20-0.93	Wang *et al.* (2013)

Table S2. Maerl beds sampled for this study grouped by region.

Region	Maerl bed	Coordinates	
British Isles (BI)	Strangford Lough (Northern Ireland)	54º 22' 42.9'' N	05º 33' 50.7'' W
	Galway (Ireland)	53º 14' 48.2'' N	09º 37' 42.4'' W
	Falmouth (England)	50º 08' 08.3'' N	05º 03' 27.1'' W
	Poole Bay (England)	50º 36' 18.1'' N	01º 52' 03.7'' W
	Milford Haven (England)	51º 42' 17.6'' N	05º 04' 56.9'' W
Atlantic France (AF)	Baie de Morlaix (Brittany)	48º 42' 41.3'' N	03º 57' 04.7'' W
	Molène Archipelago (Brittany)	48º 23' 09.0'' N	04º 51' 14.0'' W
	Glénan (Brittany)	47º 43' 14.6'' N	04º 01' 58.0'' W
	Île de Ré (Poitou-Charentes)	46º 13' 51.0'' N	01º 22' 24.0'' W
Atlantic Spain (AS)	Bornalle (Ría de Muros e Noia)	42º 47' 19.4'' N	09º 01' 11.3'' W
	Punta Barbafeita (Ría de Arousa)	42º 34' 09.9'' N	08º 53' 25.9'' W
	Nido do Corvo (Ría de Arousa)	42º 33' 42.5'' N	08º 53' 27.5'' W
	Illa Benencia (Ría de Arousa)	42º 35' 59.0'' N	08º 52' 26.0'' W
	Praia de Tulla (Ría de Pontevedra)	42º 20' 28.6'' N	08º 48' 39.1'' W
	Illa de Ons (Ría de Pontevedra)	42º 23' 40.7'' N	08º 54' 54.9'' W
	Illas Cíes (Ría de Vigo)	42º 12' 45.5'' N	08º 53' 49.0'' W

CONCLUSIONS

For the first time, the diversity of maerl-forming species in European Atlantic beds was assessed with an integrative taxonomic approach, finding 13 taxa with biologically pausible ranges (chapter 1). Among them, 6 taxa were assigned to available species names: *Lithothamion glaciale, L. corallioides, Phymatolithon calcareum*, *Mesophyllum sphaericum*, *Lithophyllum fasciculatum*, and *L. dentatum.* Recently, two of them (*L. fasciculatum* and *L. dentatum*) have been regarded as conspecifics with *Lithophyllum incrustans* after comparison with sequence data from the generitype in literature. Our molecular-assisted survey suggests that a majority of maerl-forming species have small distribution ranges, and it exists a gradual replacement of species with latitude. Likewise, cold northern latitudes are dominated by two members of genus *Lithothamnion—L. glaciale* and a still unnamed taxa (*Lithothamnion* sp.1)—whereas *L. corallio*ides and *P. calcareum* are the main constituents of maerl beds in French Brittany and English Channel. To the south, *P. calcareum* and *L. corallioides* are gradually replaced by another major maerl-forming species in Iberia, contradicting the widespread believe that only *P. calcareum* and *L. corallioides* were the main builders of maerl in European Atlantic beds south of the English Channel. Our results indicate that this newly discovered major component of maerl beds belongs to genus *Phymatolithon,* and was described as *Phymatolithon lusitanicum* sp. nov. in chapter 2.

Also for the first time, the sexual stage of *Phymatolithon calcareum* was studied with a combination of DNA barcodes and morphology. Our DNA barcodes, that include the neotype of *P. calcareum*, confirm that the gametangial stage of this algae is an encrusting plant (chapter 3). Gametangial plants of *P. calcareum* are notably hard to find and thallus fragmentation is suggested as the main method of maerl propagation. In this thesis, we investigated two collections found in a subtidal maerl in the Molène Archipelago (Brittany). Interestingly, this is the same region where literature has previously found crustose individuals identified as gametophytes of *P. calcareum* based on morphology.

We developed the first set of microsatellite markers for a coralline alga. Specifically, eight loci were isolated, and their polymorphism assessed, for *Phymatolithon calcareum* using 454 NGS technology. These eight loci were successfully incorporated into a multiplexed PCR protocol (chapter 4).

REFERENCES

Adey W. H. (1964). The genus *Phymatolithon* in the Gulf of Maine. *Hydrobiologia* 24, 377-420.

Adey W. H. (1968). The distribution of crustose corallines on the Icelandic coast. *Scientia Islandica* 1, 16-25.

Adey W. H. (1971). The sublittoral distribution of crustose corallines on the Norwegian coast. *Sarsia* 46, 41-58.

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.

Adey W. H., Chamberlain Y. M. & Irvine L. M. (2005). An SEM-based analysis of the morphology, anatomy, and reproduction of *Lithothamnion tophiforme* (Esper) Unger (Corallinales, Rhodophyta), with a comparative study of associated North Atlantic Arctic/Subarctic Melobesioideae. *Journal of Phycology* 41, 1010-1024.

Adey W. H., Hernandez-Kantun J. J., Johnson G. & Gabrielson P. W. (2015). DNA sequencing, anatomy, and calcification patterns support a monophyletic, subarctic, carbonate reef-forming *Clathromorphum* (Hapalidiaceae, Corallinales, Rhodophyta). *Journal of Phycology* 51, 189-203.

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.

Afonso-Carrillo J. & Gil-Rodríguez M. C. (1982). Sobre la presencia de un fondo de "maerl" en las Islas Canarias. *Collectanea Botanica* 13, 703-708.

Agostinho D. D. C. & Necchi O. Jr. (2014). Systematics of the section *Virescentia* of the genus *Batrachospermum* (Batrachospermales, Rhodophyta) in Brazil. *Phycologia* 53, 561-570.

Albrechtsen A., Nielsen F. C. & Nielsen R. (2010). Ascertainment biases in SNP chips affect measures of population divergence. *Molecular Biology & Evolution* 27, 2534-2547.

Allendorf F. W., Luikart G. & Aitken S. N. (2013). *Conservation and the Genetic of Populations*, 2nd edn. Wiley-Blackwell, West Sussex, UK.

Andersson L. (1990). The driving force: species concepts and ecology. *Taxon* 39, 375-382.

Andreakis N., Kooistra W. H. C. F. & Procaccini G. (2007). Microsatellite markers in an invasive strain of *Asparagopsis taxiformis* (Bonnemaisoniales, Rhodophyta): insights in ploidy level and sexual reproduction. *Gene* 406, 144-151.

Andreakis N., Kooistra W. H. C. F. & Procaccini G. (2009). High genetic diversity and connectivity in the polyploid invasive seaweed *Asparagopsis taxiformis* (Bonnemaisoniales) in the Mediterranean, explored with microsatellite alleles and multilocus genotypes. *Molecular Ecology* 18, 212-226.

Anthony K. R. N., Kline D. I., Diaz Pulido G., Dove S. & Hoegh-Guldberg O. (2008). Ocean acidification causes bleaching and productivity loss in coral reef builders. *Proceedings of the National Academy of Sciences of the United States of America* 105, 17442-17446.

Aranda S. C., Gradstein S. R., Patiño J., Laenen B., Désamoré A. & Vanderpoorten A. (2014). Phylogeny, classification and species delimitation in the liverwort genus *Odontoschisma* (Cephaloziaceae). *Taxon* 63, 1008-1025.

Ardré F. (1970). Contribution à l'étude des algues marines du Portugal. I. La Flore. *Portugaliae Acta Biologica, Série B.* 10, 137-555.

Arif I. A., Khan H. A., Bahkali A. H., Al Homaidan A. A., Al Farhan A. H., Al Sadoon M. & Shobrak M. (2011). DNA marker technology for wildlife conservation. *Saudi Journal of Biological Sciences* 18, 219-225.

Arnaud-Haond S., Alberto F., Teixeira S., Procaccini G., Serrão E. A. & Duarte C. M. (2005). Assessing genetic diversity in clonal organisms: low diversity or low resolution? Combining power and cost efficiency in selecting markers. *Journal of Heredity* 96, 434-440.

Arnaud-Haond S. & Belkhir K. (2007). GENCLONE: a computer program to analyse genotypic data, test for clonality and describe spatial clonal organization. *Molecular Ecology Notes* 7, 15-17.

Arnaud-Haond S., Duarte C. M., Alberto F. & Serrão E. A. (2007). Standardizing methods to address clonality in population studies. *Molecular Ecology* 16, 5115-5139.

Arnaud-Haond S., Marbà N., Diaz-Almela E., Serrão E. A. & Duarte C. M. (2010). Comparative analysis of stability—genetic diversity in seagrass (*Posidonia oceanica*) meadows yields unexpected results. *Estuaries & Coasts* 33, 878-889.

Aydin Z., Marcussen T., Ertekin A. S. & Oxelman B. (2014). Marginal likelihood estimate comparisons to obtain optimal species delimitations in *Silene* sect. *cryptoneurae* (Caryophyllaceae). *PLoS ONE* 9, e106990 (p. 9).

Ayres-Ostrock L. M., Mauger S., Plastino E. M., Oliveira M. C., Valero M. & Destombe C. (2015). Development and characterization of microsatellite markers in two agarophyte species, *Gracilaria birdiae* and *Gracilaria caudata* (Gracilariaceae, Rhodophyta), using next-generation sequencing. *Journal of*

Applied Phycology, First online: 23 April 2015, 1-10.

Babbini L. & Bressan G. (1997). *Recensement de Corallinacées de la Mer Méditerranée et considérations phytogéographiques.* Bibliotheca Phycologica, Berlin & Stuttgart, Germany.

Baele G., Lemey P., Bedford T., Rambaut A., Suchard M. A. & Alekseyenko A. V. (2012). Improving the accuracy of demographic and molecular clock model comparison while accommodating phylogenetic uncertainty. *Molecular Biology & Evolution* 29, 2157-2167.

Baird N. A., Etter P. D., Atwood T. S., Currey M. C., Shiver A. L., Lewis Z. A., Selker E. U., Cresko W. A. & Johnson E. A. (2008). Rapid SNP Discovery and genetic mapping using sequenced RAD markers. *PLoS ONE* 3, e3376 (p. 7).

Ballesteros E. (1988). Composición y estructura de los fondos de maërl de Tossa de Mar (Gerona, España). *Collectanea Botanica* 17, 161-182.

Balloux F., Lehmann L. & de Meeûs T. (2003). The population genetics of clonal and partially clonal diploids. *Genetics* 164, 1635-1644.

Bárbara I., Calvo S., Cremades J., Díaz P., Dosil Mancilla F. X., Peña V., López Varela C., Novo T., Veiga A. J. & López Rodríguez M. C. (2003). Fragmenta Chorologica Occidentalia, Algae, 8641-8747. *Anales del Jardín Botánico de Madrid* 60, 409-416.

Barrett S. C. H., Eckert C. G. & Husband B. C. (1993). Evolutionary processes in aquatic plant populations. *Aquatic Botany* 44, 105-145.

Bartsch I., Wiencke C. & Laepple T. (2012). Global Seaweed Biogeography Under a Changing Climate: The Prospected Effects of Temperature. In: *Seaweed Biology* (eds. Wiencke C. & Bischof K.), pp. 383-406. Springer Berlin Heidelberg, Berlin, Germany.

Basso D., Nalin R. & Nelson C. (2009). Shallow-water *Sporolithon* rhodoliths from North Island (New Zealand). *Palaios* 24, 92-103.

Bauch R. (1937). Die entwicklung der bisporen der Corallinaceen. *Planta* 26, 365-390.

Becheler R., Benkara E., Moalic Y., Hily C. & Arnaud-Haond S. (2014a). Scaling of processes shaping the clonal dynamics and genetic mosaic of seagrasses through temporal genetic monitoring. *Heredity* 112, 114-121.

Becheler R., Diekmann O., Hily C., Moalic Y. & Arnaud-Haond S. (2014b). The concept of population in clonal organisms: mosaics of temporally colonized patches are forming highly diverse meadows of *Zostera marina* a in Brittany. Erratum. *Molecular Ecology* 23, 2886-2886.

Becheler R., Diekmann O., Hily C., Moalic Y. & Arnaud Haond S. (2010). The concept of population in clonal organisms: mosaics of temporally colonized patches are forming highly diverse meadows of *Zostera marina* in Brittany. *Molecular Ecology* 19, 2394-2407.

Berthouly-Salazar C., Cassey P., van Vuuren B. J., van Rensburg B. J., Hui C., Gardner M. G. & Le Roux J. J. (2012). Development and characterization of 13 new, and cross amplification of 3, polymorphic nuclear microsatellite loci in the common myna (*Acridotheres tristis*). *Conservation Genetics Resources* 4, 621-624.

Bhargava A. & Fuentes F. F. (2010). Mutational dynamics of microsatellites. *Molecular Biotechnology* 44, 250-266.

Bi Y.-H., Wu Y.-Y. & Zhou Z.-G. (2014). Genetic diversity of wild population of *Pyropia haitanensis* based on SSR analysis. *Biochemical Systematics & Ecology* 54, 307-312.

Bi Y., Yang X., Sun Z. & Zhou Z. (2015). Development and characterization of 12 polymorphic microsatellite markers in *Sargassum vachellianum*. *Conservation Genetics Resources* 7, 203-205.

Billot C., Rousvoal S., Estoup A., Epplen J. T., Saumitou-Laprade P., Valero M. & Kloareg B. (1998). Isolation and characterization of microsatellite markers in the nuclear genome of the brown alga *Laminaria digitata* (Phaeophyceae). *Molecular Ecology* 7, 1778-1780.

Binks R. M., Millar M. A. & Byrne M. (2015). Contrasting patterns of clonality and fine-scale genetic structure in two rare sedges with differing geographic distributions. *Heredity* 115, 235-242.

BIOMAERL Team (2003). Conservation and management of northeast Atlantic and Mediterranean maerl beds. *Aquatic Conservation: Marine & Freshwater Ecosystems* 13, S65-S76.

Birkett D. A., Maggs C. & Dring M. J. (1998). Maerl (volume V). An overview of dynamics and sensitivity characteristics for conservation management of marine SACs, p. 116. Scottish Association for Marine Science, UK Marine Special Areas of Conservation Project.

Birky C. W. Jr. (2013). Species detection and identification in sexual organisms using population genetic theory and DNA sequences. *PLoS ONE* 8, e52544 (p. 11).

Birky C. W. Jr., Adams J., Gemmel M. & Perry J. (2010). Using population genetic theory and DNA sequences for species detection and identification in asexual organisms. *PLoS ONE* 5, e10609 (p. 11).

Bittner L. (2009). Phylogénie des Corallinales (Rhodophyta) et analyse de leur diversité génétique dans le Pacifique Sud. PhD thesis,

Muséum National d´Histoire Naturelle, Paris, France.

Bittner L., Halary S., Payri C., Cruaud C., de Reviers B., Lopez P. & Bapteste E. (2010). Some considerations for analyzing biodiversity using integrative metagenomics and gene networks. *Biology Direct* 5, 47 (p. 17).

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 & Evolution* 61, 697-713.

Blunden G., Farnham W. F., Jephson N., Barwell C. J., Fenn R. H. & Plunkett B. A. (1981). The composition of maërl beds of economic interest in Northern Brittany, Cornwall and Ireland. *Xth International Seaweed Symposium* 10, 651-656.

Bosence D. W. (1976). Ecological studies on two unattached coralline algae from western Ireland. *Palaeontology* 19, 365-395.

Bostein D., White R. L., Skolnick M. & Davis R. W. (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *American Journal of Human Genetics* 32, 314-331.

Bressan G. & Babbini L. (2003). Biodiversità Marina delle Coste Italiane: Corallinales del mar Mediterraneo: guida alla determinazione. *Biologia Marina Mediterranea* 10, 1-237.

Briand X. (1991). Seaweed Harvesting in Europe. In: *Seaweed Resources in Europe: Uses and Potential*, pp. 259-308. John Wiley & Sons, West Sussex, UK.

Brodie J., Hayes P. K., Barker G. L. & Irvine L. M. (1996). Molecular and morphological characters distinguishing two *Porphyra* species (Rhodophyta: Bangiophycidae). *European Journal of Phycology* 31, 303-308.

Brodie J., Hayes P. K., Barker G. L., Irvine L. M. & Bartsch I. (1998). A reapraisal of *Porphyra* and *Bangia* (Bangiophycidae, Rhodophyta) in the Northeast Atlantic based on the *rbc*L-*rbc*S intergeneic spacer. *Journal of Phycology* 34, 1069-1074.

Brodie J., Williamson C., Smale D. A., Kamenos N. A., Mieszkowska N., Santos R., Cunliffe M., Steinke M., Yesson C., Anderson K. M., Asnaghi V., Brownlee C., Burdett H. L., Burrows M. T., Collins S., Donohue P. J. C., Harvey B., Foggo A., Noisette F., Nunes J., Ragazzola F., Raven J. A., Schmidt D. N., Suggett D., Teichberg M. & Hall Spencer J. M. (2014). The future of the northeast Atlantic benthic flora in a high CO_2 world. *Ecology & Evolution* 4, 2787-2798.

Brown S. D. J., Collins R. A., Boyer S., Lefort M.-C., Malumbres-Olarte J., Vink C. J. & Cruickshank R. H. (2012). SPIDER: an R package for the analysis of species identity and evolution, with particular reference to DNA barcoding. *Molecular Ecology Resources* 12, 562-565.

Büdenbender J., Riebesell U. & Form A. (2011). Calcification of the Arctic coralline red algae *Lithothamnion glaciale* in response to elevated CO_2. *Marine Ecology Progress Series* 441, 79-87.

Burdett H. L., Aloisio E., Calosi P., Findlay H. S., Widdicombe S., Hatton A. D. & Kamenos N. A. (2012). The effect of chronic and acute low pH on the intracellular DMSP production and epithelial cell morphology of red coralline algae. *Marine Biology Research* 8, 756-763.

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.

Cabioch J. (1969). Les fonds de maerl de la Baie de Morlaix et leur peuplement végétal. *Cahiers de Biologie Marine* 10, 139-161.

Cabioch J. (1970). Le maërl des côtes de Bretagne et le probléme de sa survie. *Penn ar Bed* 7, 421-429.

Cabioch J. (1972). Étude sur les Corallinacées. II. La morphogenèse; conséquences systématiques et phylogénétiques. *Cahiers de Biologie Marine* 13, 137-288.

Cabioch J. (1974). Un fond de maerl de l'Archipel de Madère et son peuplement végétal. *Bulletin de la Société Phycologique de la France* 19, 74-82.

Carro B., Lopez L., Peña V., Bárbara I. & Barreiro R. (2014). DNA barcoding allows the accurate assessment of European maerl diversity: a proof-of-concept study. *Phytotaxa* 190, 176-189.

Carstens B. C. & Dewey T. A. (2010). Species delimitation using a combined coalescent and information-theoretic approach: an example from North American *Myotis* bats. *Systematic Biology* 59, 400-414.

Carstens B. C., Pelletier T. A., Reid N. M. & Satler J. D. (2013). How to fail at species delimitation. *Molecular Ecology* 22, 4369-4383.

Castoe T. A., Poole A. W., Gu W., de Koning A. P. J., Daza J. M., Smith E. N. & Pollock D. D. (2010). Rapid identification of thousands of copperhead snake (*Agkistrodon contortrix*) microsatellite loci from modest amounts of 454 shotgun genome sequence. *Molecular Ecology Resources* 10, 341-347.

Ceccarelli F. S., Menegon M., Tolley K. A., Tilbury C. R., Gower D. J., Laserna M. H., Kasahun R., Rodriguez-Prieto A., Hagmann R. & Loader S. P. (2014). Evolutionary relationships, species delimitation and biogeography of Eastern Afromontane horned chameleons (Chamaeleonidae: *Trioceros*). *Molecular Phylogenetics & Evolution* 80, 125-136.

Cecere E., Petrocelli A. & Verlaque M. (2011). Vegetative reproduction by multicellular propagules in Rhodophyta: an overview. *Marine Ecology* 32, 419-437.

Chamberlain Y. M. (1990). The genus *Leptophytum* (Rhodophyta, Corallinaceae) in the British Isles with descriptions of *Leptophytum bornetii*, *L. elatum* sp. nov. and *L. laeve*. *British Phycological Journal* 25, 179-199.

Chamberlain Y. M. (1991). Observations on *Phymatolithon lamii* (Lemoine) Y. Chamberlain comb. nov. (Rhodophyta, Corallinales) in the British Isles with an assessment of its relationship to *P. rugulosum*, *Lithophyllum lamii* and *L. melobesioides*. *British Phycological Journal* 26, 219-233.

Chamberlain Y. M. & Irvine L. M. (1994). MELOBESIOIDEAE Bizzozero. In: *Seaweeds of the British Isles. Volume 1. Rhodophyta Part 2B Corallinales, Hildenbrandiales* (eds. Irvine L. M. & Chamberlain Y. M.), pp. 159-234. The Natural History Museum, London, UK.

Chaotian X., Changsheng C., Yan X. & Dehue J. (2010). Construction of a genetic linkage map for *Porphyra haitanensis* (Bangiales, Rhodophyta) based on sequence-related amplified polymorphism and simple sequence repeat markers. *Journal of Phycology* 46, 780-787.

Chen X., Jiang K., Guo P., Huang S., Rao D., Ding L., Takeuchi H., Che J., Zhang Y.-p., Myers E. A. & Burbrink F. T. (2014a). Assessing species boundaries and the phylogenetic position of the rare Szechwan ratsnake, *Euprepiophis perlaceus* (Serpentes: Colubridae), using coalescent-based methods. *Molecular Phylogenetics & Evolution* 70, 130-136.

Chen X., Xu S., Yu Z., Guo L., Yang S., Liu L., Yang X. & Liu J. (2014b). Multiple lines of evidence on the genetic relatedness of the parthenogenetic and bisexual *Haemaphysalis longicornis* (Acari: Ixodidae). *Infection, Genetics & Evolution* 21, 308-314.

Clarkston B. E. & Saunders G. W. (2010). A comparison of two DNA barcode markers for species discrimination in the red algal family Kallymeniaceae (Gigartinales, Florideophyceae), with a description of *Euthora timburtonii* sp nov. *Botany* 88, 119-131.

Clarkston B. E. & Saunders G. W. (2012). An examination of the red algal genus *Pugetia* (Kallymeniaceae, Gigartinales), with descriptions of *Salishia firma* gen. & comb. nov., *Pugetia cryptica* sp. nov. and *Beringia wynnei* sp. nov. *Phycologia* 51, 33-61.

Clayden S. L. & Saunders G. W. (2010). Recognition of *Rubrointrusa membranacea* gen. et comb. nov., *Rhodonematella subimmersa* gen. et comb. nov. (with a reinterpretation of the life history) and the Meiodiscaceae fam. nov. within the Palmariales (Rhodophyta). *Phycologia* 49, 283-300.

Clayden S. L. & Saunders G. W. (2014). A study of two *Acrochaetium* complexes in Canada with distinction of *Rhododrewia* gen. nov. (Acrochaetiales, Rhodophyta). *Phycologia* 53, 221-232.

Clisson I., Lathuilliere M. & Croau-Roy B. (2000). Conservation and evolution of microsatellite loci in primate taxa. *American Journal of Primatology* 50, 205-214.

Coelho N. C., Serrão E. A. & Alberto F. (2014). Characterization of fifteen microsatellite markers for the kelp *Laminaria ochroleuca* and cross species amplification within the genus. *Conservation Genetics Resources* 6, 949-950.

Coleman M., Dolman G., Kelaher B. P. & Steinberg P. D. (2008). Characterisation of microsatellite loci in the subtidal habitat-forming alga, *Phyllospora comosa* (Phaeophyceae, Fucales). *Conservation Genetics* 9, 1015-1017.

Collens J. D. (2009). Isolation and description of eight polymorphic microsatellite loci for the winged-kelp *Alaria nana*. *Molecular Ecology Resources* 9, 1421-1423.

Conklin K. Y., Kurihara A. & Sherwood A. R. (2009). A molecular method for identification of the morphologically plastic invasive algal genera *Eucheuma* and *Kappaphycus* (Rhodophyta, Gigartinales) in Hawaii. *Journal of Applied Phycology* 21, 691-699.

Conklin K. Y., O'Doherty D. C. & Sherwood A. R. (2014). *Hydropuntia perplexa* comb. nov. (Gracilariaceae, Rhodophyta), first record of the genus in Hawai'i. *Pacific Science* 68, 421-434.

Coppejans E. (1978). Sur les propagules de *Fosliella farinosa* (Lamouroux) Howe var. *farinosa* (Rhodophyceae-Cryptonemiales). *Bulletin de la Société Royale de Botanique de Belgique* 111, 55-61.

Costa E. S., Plastino E. M., Petti R., Oliveira E. C. & Oliveira M. C. (2012). The Gracilariaceae germplasm bank of the University of São Paulo, Brazil—a DNA barcoding approach. *Journal of Applied Phycology* 24, 1643-1653.

Couceiro L., Maneiro I., Mauger S., Valero M., Ruiz J. M. & Barreiro R. (2011a). Microsatellite development in Rhodophyta using high-throughput sequence data. *Journal of Phycology* 47, 1258-1265.

Couceiro L., Maneiro I., Ruiz J. M. & Barreiro R. (2011b). Multiscale genetic of an endangered seaweed *Ahnfeltiopsis pusilla* (Rhodophyta): implications for its conservation. *Journal of Phycology* 47, 259-268.

Coyer J. A., Hoarau G., Beszteri B., Pearson G. & Olsen J. L. (2009). Expressed sequence tag-derived polymorphic SSR markers for *Fucus serratus* and amplification in other species of *Fucus*. *Molecular Ecology Resources* 9, 168-170.

Coyer J. A., Veldsink J. H., Jones K., Stam W. T. & Olsen J. L. (2002). Characterization of microsatellite loci in the marine seaweeds, *Fucus serratus* and *F. evanescens* (Heterokontophyta; Fucaceae). *Molecular Ecology Notes* 2, 35-37.

Cronquist A. (1978). Once Again, What Is a Species? In: *Biosystematics in Agriculture* (ed. Romberger J. A.), pp. 3-20. Allenheld & Osmin, Montclair, New Jersey, USA.

Crouan P. L. & Crouan H. M. (1867). *Florule du Finistère*. Friedrich Klincksieck & J. B. et A. Lefournier, Paris & Brest, France.

Daguin C., Voisin M., Engel C. & Viard F. (2006). Microsatellites isolation and polymorphism in introduced populations of the cultivated seaweed *Undaria pinnatifida* (Phaeophyceae, Laminariales). *Conservation Genetics* 6, 647-650.

Darriba D., Taboada G. L., Doallo R. & Posada D. (2012). jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods* 9, 772-772.

de Jesus P. B., Silva M. S., Lyra G. d. M., Nunes J. M. d. C. & Schnadelbach A. S. (2015). Extension of the distribution range of *Hypnea stellulifera* (Cystocloniaceae, Rhodophyta) to the South Atlantic: morphological and molecular evidence. *Aquatic Botany* 123, 26-36.

de Queiroz K. (2005). Ernst Mayr and the modern concept of species. *Proceedings of the National Academy of Sciences of the United States of America* 102 (S1), 6600-6607.

de Queiroz K. (2007). Species concepts and species delimitation. *Systematic Biology* 56, 879-886.

de Salle R. (2006). Species discovery *versus* species identification in DNA barcoding efforts: response to Rubinoff. *Conservation Biology* 20, 1545-1547.

Dixon K. R. & Saunders G. W. (2013). DNA barcoding and phylogenetics of *Ramicrusta* and *Incendia* gen. nov., two early diverging lineages of the Peyssonneliaceae (Rhodophyta). *Phycologia* 52, 82-108.

Dolman G. & Coleman M. A. (2009). Characterisation of microsatellite loci in the habitat-forming kelp, *Ecklonia radiata* (Phaeophyceae, Laminariales). *Conservation Genetics* 10, 657-660.

Dolms-Lasubach H. (1881). Die Corallinenalgen Des Golfes Von Neapel Und Der Angrenzenden Meeres-Abschnitte: Eine Monographie. In: *Fauna und Flora des Golfes von Neapel und der angrenzenden Meeres-abschnitte. Herausgegeben von der Zoologischen Station zu Neapel. 18 Monographie*, pp. 1-64. Leipzig, W. Engelmann, Berlin, Germany.

Donoghue M. (1985). A critique of the biological species concept and recommendations for a phylogenetic alternative. *The Bryologist* 88, 172-181.

Dorken M. E. & Eckert C. G. (2001). Severely reduced sexual reproduction in northern populations of a clonal plant, *Decodon verticillatus* (Lythraceae). *Journal of Ecology* 89, 339-350.

Drummond A., Suchard M. A., Xie D. & Rambaut A. (2012). Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology & Evolution* 29, 1969-1973.

Dupuis J. R., Roe A. D. & Sperling F. a. H. (2012). Multi-locus species delimitation in closely related animals and fungi: one marker is not enough. *Molecular Ecology* 21, 4422-4436.

Eckert C. G. (2002). The loss of sex in clonal plants. *Evolutionary Ecology* 15, 501-520.

Eckert C. G. & Barrett S. C. H. (1993). Clonal reproduction and patterns of genotypic diversity in *Decodon verticillatus* (Lythraceae). *American Journal of Botany* 80, 1175-1182.

Edyvean R. G. J. & Ford H. (1986). Spore production by *Lithophyllum incrustans* (Corallinales, Rhodophyta) in the British Isles. *British Phycological Journal* 21, 255-261.

Ellegren H. (2004). Microsatellites: simple sequences with complex evolution. *Nature Reviews, Genetics* 5, 435-445.

Ence D. D. & Carstens B. C. (2011). SpedeSTEM: a rapid and accurate method for species delimitation. *Molecular Ecology Resources* 11, 473-480.

Engel C. R., Brawley S. H., Edwards K. J. & Serrão E. (2003). Isolation and cross-species amplification of microsatellite loci from the fucoid seaweeds *Fucus vesiculosus*, *F. serratus* and *Ascophyllum nodosum* (Heterokontophyta, Fucaceae). *Molecular Ecology Notes* 3, 180-182.

Engel C. R., Destombe C. & Valero M. (2004). Mating system and gene flow in the red seaweed *Gracilaria gracilis*: effect of haploid-diploid life history and intertidal rocky shore landscape on fine-scale genetic structure. *Heredity* 92, 289-298.

Engel C. R., Guillemin M. L., Jacob A. M., Valero M. & Viard F. (2008). Isolation of microsatellite loci from the kelp, *Saccorhiza polyschides* (Heterokontophyta, *incertae sedis*). *Molecular Ecology Resources* 8, 406-408.

Eriksson O. (1993). Dynamics of genets in clonal plants. *Trends in Ecology & Evolution* 8, 313-316.

Farr T., Broom J., Hart D., Neill K. & Nelson W. (2009). *Common Coralline Algae of Northern New Zealand: An Identification Guide*. NIWA Information Series nº 70, Wellington, New Zealand.

Faugeron S., Veliz D., Peralta G., Tapia J., Tellier F., Billot C. & Martinez E. (2009). Development and characterization of nine polymorphic microsatellite markers in the Chilean kelp *Lessonia nigrescens*. *Molecular Ecology Resources* 9, 937-939.

Fontaneto D., Herniou E. A., Boschetti C., Caprioli M., Melone G., Ricci C. & Barraclough T. G. (2007). Independently evolving species in asexual bdelloid rotifers. *PLoS Biology* 5, e87 (p. 8).

Foslie M. (1895, reprinted from 1894). The Norwegian Forms of *Lithothamnion. Det Kongelige Norske Videnskabernes Selskab Skrifter* (1894) 1, 29-208.

Foslie M. (1899). A visit to Roundstone in April. *The Irish Naturalist* 8, 175-180.

Foslie M. (1900). New or critical calcareous algae. *Det Kongelige Norske Videnskabernes Selskab Skrifter* (1899) 5, 1-34.

Foslie M. (1905). *Remarks on Northern Lithothamnia*. *Det Kongelige Norske Videnskabernes Selskab Skrifter* 3, 1-138.

Foslie M. (1906). Algologiske notiser II. *Det Kongelige Norske Videnskabernes Selskab Skrifter* 1, 1-28.

Foslie M. (1908). Die Lithothamnien der Deutschen Südpolar-Expedition 1901-1903 / von M. Foslie. Studies of *Lithothamnia*. *Deutsche Südpolar-Expedition (1901-1903)* 8, 205-219.

Foslie M. & Printz H. (1929). *Contributions to a Monograph of the Lithothamnia*. Det Kongelige Norske Videnskabers Selskab Museet, Trondhjem, Norway.

Foster M. (2001). Rhodoliths: between rocks and soft places. *Journal of Phycology* 37, 659-667.

Frankham R. (1996). Relationship of genetic variation to population size in wildlife. *Conservation Biology* 10, 1500-1508.

Frankham R., Ballou J., D. & Briscoe D. A. (2002). *Introduction to Conservation Genetics*, 5th edn. Cambridge University Press, Cambridge, UK.

Frankham R., Ballou J., D. & Briscoe D. A. (2004). *A Primer Conservation Genetics.* Cambridge University Press, New York, USA.

Freiwald A. (1995). Sedimentological and biological aspects in the formation of branched rhodoliths in northern Norway. *Beiträge zur Paläontologie* 20, 7-19.

Fujisawa T. & Barraclough T. G. (2013). Delimiting species using single-locus data and the generalized mixed Yule coalescent approach: a revised method and evaluation on simulated data sets. *Systematic Biology* 62, 707-724.

Fujita M. K., Leaché A. D., Burbrink F. T., McGuire J. A. & Moritz C. (2012). Coalescent-based species delimitation in an integrative taxonomy. *Trends in Ecology & Evolution* 27, 480-488.

Gardner M. G., Fitch A. J., Bertozzi T. & Lowe A. (2011). Rise of the machines – recommendations for ecologists when using next generation sequencing for microsatellite development. *Molecular Ecology Resources* 11, 1093–1101.

Gaston K. J. (1996). Species-range-size distributions: patterns, mechanisms and implications. *Trends in Ecology & Evolution* 11, 197-201.

Gaylord B., Reed D. C., Raimondi P. T., Washburn L. & McLean S. R. (2002). A physically based model of macroalgal spore dispersal in the wave and current-dominated nearshore. *Ecology* 83, 1239-1251.

Genovese G., Faggio C., Gugliandolo C., Torre A., Spanò A., Morabito M. & Maugeri T. L. (2012). *In vitro* evaluation of antibacterial activity of *Asparagopsis taxiformis* from the Straits of Messina against pathogens relevant in aquaculture. *Marine Environmental Research* 73, 1-6.

Geoffroy A., Le Gall L. & Destombe C. (2012). Cryptic introduction of the red alga *Polysiphonia morrowii* Harvey (Rhodomelaceae, Rhodophyta) in the North Atlantic Ocean highlighted by a DNA barcoding approach. *Aquatic Botany* 100, 67-71.

Goldstein D. B. & Schlotterer C. (1999). *Microsatellites: Evolution and Applications.* Oxford University Press, Oxford, UK.

Gouy M., Guindon S. & Gascuel O. (2010). SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Molecular Biology & Evolution* 27, 221-224.

Grall J. (2003). Fiche de synthése sur les biocénoses: le bancs de maërl, p. 20. REBENT, Réseau Benthique, IFREMER, France.

Grall J. & Hall-Spencer J. M. (2003). Problems facing maerl conservation in Brittany. *Aquatic Conservation: Marine & Freshwater Ecosystems* 13, S55-S64.

Grummer J. A., Bryson R. W. Jr. & Reeder T. W. (2014). Species delimitation using Bayes Factors: simulations and application to the *Sceloporus*

scalaris species group (Squamata: Phrynosomatidae). *Systematic Biology* 63, 119-133.

Guichoux E., Lagache L., Wagner S., Chaumeil P., Léger P., Lepais O., Lepoittevin C., Malausa T., Revardel E., Salin F. & Petit R. J. (2011). Current trends in microsatellite genotyping. *Molecular Ecology Resources* 11, 591-611.

Guillemin M.-L., Faugeron S., Destombe C., Viard F., Correa J. & Valero M. (2008). Genetic variation in wild and cultivated populations of the haploid-diploid red alga *Gracilaria chilensis*: how farming practices favor asexual reproduction and heterozygosity. *Evolution* 62, 1500-1519.

Guillemin M. L., Destombe C., Faugeron S., Correa J. A. & Valero M. (2005). Development of microsatellites DNA markers in the cultivated seaweed, *Gracilaria chilensis* (Gracilariales, Rhodophyta). *Molecular Ecology Notes* 5, 155-157.

Guindon S. & Gascuel O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* 52, 696-704.

Guiry M. D. (1978). *A consensus and bibliography of Irish Seaweeds.* J. Cramer, Vaduz, Liechtenstein.

Guiry M. D. (1990). Sporangia and Spores. In: *Biology of the Red Algae*, pp. 347-376. Cambridge University Press, Cambridge, UK.

Guiry M. D. & Guiry G. M. (2015). AlgaeBase. World-wide electronic publication, National University of Ireland, Galway, Ireland. http://www.algaebase.org; searched on 08 June 2015.

Gunnarsson K. (1977). Pörungar á kóralsetlögum í Arnarfirði. *Sérprentun úr Hafrannsóknir* 10, 1-10.

Guoying D., Feifei W., Hao G., Hongfan X. & Yunxiang M. (2015). DNA barcode assessment of Ceramiales (Rhodophyta) in the intertidal zone of the northwestern Yellow Sea. *Chinese Journal of Oceanology & Limnology* 33, 685-695.

Halkett F., Simon J.-C. & Balloux F. (2005). Tackling the population genetics of clonal and partially clonal organisms. *Trends in Ecology & Evolution* 20, 194-201.

Hall-Spencer J. M., Kelly J. & Maggs C. A. (2010). Background document for maërl beds. In: *OSPAR Commission Biodiversity Series*, pp. 1-36. OSPAR Commission.

Hall-Spencer J. M. & Moore P. G. (2000). Scallop dredging has profound, long-term impacts on mael habitats. *ICES Journal of Marine Sciences* 57, 1407-1415.

Hamilton C. A., Hendrixson B. E., Brewer M. S. & Bond J. E. (2014). An evaluation of sampling effects on multiple DNA barcoding methods leads to an integrative approach for delimiting species: a case study of the North American tarantula genus *Aphonopelma* (Araneae, Mygalomorphae, Theraphosidae). *Molecular Phylogenetics & Evolution* 71, 79-93.

Harada Y., Kawano S. & Iwasa Y. (1997). Probability of clonal identity: inferring the relative success of sexual *versus* clonal reproduction from spatial genetic patterns. *Journal of Ecology* 85, 591-600.

Harvey A., Woelkerling W., Farr T., Neill K. & Nelson W. (2005). *Coralline algae of cetral New Zealand. An identification guide to common "crustose" species.* NIWA Information Series No. 57, Wellington, New Zealand.

Hebert P. D. N., Cywinska A., Ball S. L. & deWaard J. R. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences* 270, 313-321.

Hebert P. D. N., Stoeckle M. Y., Zemlak T. S. & Francis C. M. (2004). Identification of birds through DNA barcodes. *PLoS Biology* 2, e312 (p.7).

Heesch S., Cho G. Y., Peters A. F., Le Corguille G., Falentin C., Le Corguillé G., Boutet G., Coëdel S., Jubin C., Samson G., Corre E., Coelho S. M. & Cock J. M. (2010). A sequence-tagged genetic map for the brown alga *Ectocarpus siliculosus* provides large-scale assembly of the genome sequence. *New Phytologist* 188, 42-51.

Heled J. & Drummond A. J. (2010). Bayesian inference of species trees from multilocus data. *Molecular Biology & Evolution* 27, 570-580.

Hernandez-Kantun J. J., Rindi F., Adey W. H., Heesch S., Peña V., Le Gall L. & Gabrielson P. W. (2015a). Sequencing type material resolves the identity and distribution of the generitype *Lithophyllum incrustans*, and related European species *L. hibernicum* and *L. bathyporum* (Corallinales, Rhodophyta). *Journal of Phycology* 51, 791–807.

Hernández-Kantún J. J., Riosmena-Rodriguez R., Adey W. H. & Rindi F. (2014). Analysis of the *cox*2-3 spacer region for population diversity and taxonomic implications in rhodolith-forming species (Rhodophyta: Corallinales). *Phytotaxa* 190, 331-354.

Hernández-Kantún J. J., Riosmena-Rodriguez R., Hall-Spencer J. M., Peña V., Maggs C. A. & Rindi F. (2015b). Phylogenetic analysis of rhodolith formation in the Corallinales (Rhodophyta). *European Journal of Phycology* 50, 46-61.

Herren L. W., Walters L. J. & Beach K. S. (2013). Fragment production and recruitment ecology of the red alga *Laurencia poiteaui* in Florida

Bay, USA. *Journal of Experimental Marine Biology & Ecology* 440, 192-199.

Hind K. R., Gabrielson P. W., Lindstrom S. C. & Martone P. T. (2014a). Misleading morphologies and the importance of sequencing type specimens for resolving coralline taxonomy (Corallinales, Rhodophyta): *Pachyarthron cretaceum* is *Corallina officinalis*. *Journal of Phycology* 50, 760-764.

Hind K. R., Gabrielson P. W. & Saunders G. W. (2014b). Molecular-assisted alpha taxonomy reveals pseudocryptic diversity among species of *Bossiella* (Corallinales, Rhodophyta) in the eastern Pacific Ocean. *Phycologia* 53, 443-456.

Hind K. R. & Saunders G. W. (2013a). 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.

Hind K. R. & Saunders G. W. (2013b). Molecular markers from three organellar genomes unravel complex taxonomic relationships within the coralline algal genus *Chiharaea* (Corallinales, Rhodophyta). *Molecular Phylogenetics & Evolution* 67, 529-540.

Hoffman J. I., Simpson F., David P., Rijks J. M., Kuiken T., Thorne M. A. S., Lacy R. C. & Dasmahapatra K. K. (2014). High-throughput sequencing reveals inbreeding depression in a natural population. *Proceedings of the National Academy of Sciences of the United States of America* 111, 3775-3780.

Holleley C. E. & Geerts P. G. (2009). Multiplex Manager 1.0: a cross-platform computer program that plans and optimizes multiplex PCR. *BioTechniques* 46, 511-517.

Honnay O. & Bossuyt B. (2005). Prolonged clonal growth: escape route or route to extinction? *Oikos* 108, 427-432.

Hu Z.-h., Zhou Z.-g. & Yan X.-h. (2006). Isolation of microsatellite DNA in *Porphyra haitanensis*. *Marine Sciences* 30, 17-22.

Hu Z. M., Li W., Li J. J. & Duan D. L. (2011). Post-Pleistocene demographic history of the North Atlantic endemic Irish moss *Chondrus crispus*: glacial survival, spatial expansion and gene flow. *Journal of Evolutionary Biology* 24, 505-517.

Hughey J. R., Silva P. C. & Hommersand M. H. (2001). Solving taxonomic and nomenclatural problems in Pacific Gigartinaceae (Rhodophyta) using DNA from type material. *Journal of Phycology* 37, 1091-1109.

ICN (2012). *International Code of Nomenclature for algae, fungi, and plants (Melbourne Code)*. Koeltz Scientific Books, Koenigstein, Germany.

IPCC (2007). Climate Change 2007: Synthesis Report, Contribution of Working Groups I, II and III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change eds. Core Writing Team, Pachauri, R. K. & Reisinger A.), p. 104. IPCC, Geneva, Switzerland.

Irvine L. M. & Chamberlain Y. M. (1994). *Seaweeds of the British Isles. Volume 1. Rhodophyta, Part 2B Corallinales, Hildenbrandiales.* The Natural History Museum, London, UK.

Itou T., Kanno M., Suyama Y., Kamiyama A., Sakamoto S. X., Kijima A., Inaba K. & Aoki M. N. (2012). Development of 12 polymorphic microsatellite DNA markers for the kelp *Ecklonia cava* (Phaeophyceae, Laminariales). *Conservation Genetics Resources* 4, 459-461.

Janouškovec J., Liu S.-L., Martone P. T., Carré W., Leblanc C., Collén J. & Keeling P. J. (2013). Evolution of red algal plastid genomes: ancient architectures, introns, horizontal gene transfer, and taxonomic utility of plastid markers. *PLoS ONE* 8, e59001 (p. 11).

Jarne P. & Lagoda P. J. L. (1996). Microsatellites, from molecules to populations and back. *Trends in Ecology & Evolution* 11, 424-429.

Jin L., Macaubas C., Hallmayer J., Kimura A. & Mignot E. (1996). Mutation rate varies among alleles at a microsatellite locus: phylogenetic evidence. *Proceedings of the National Academy of Sciences of the United States of America* 93, 15285-15288.

Johansen H. W. (1981). *Coralline algae, A First Synthesis.* CRC Press, Boca Raton, Florida, USA.

Jokiel P. L., Rodgers K. S., Kuffner I. B., Andersson A. J., Cox E. F. & Mackenzie F. T. (2008). Ocean acidification and calcifying reef organisms: a mesocosm investigation. *Coral Reefs* 27, 473-483.

Jones L. A., Hiscock K. & Connor D. W. (2000). Marine habitat reviews. A summary of ecological requirements and sensitivity characteristics for the conservation and management of marine SACs., p. 178. UK Marine SACs Project report, Joint Nature Conservation Committee, Peterborough, UK.

Kale S. M., Pardeshi V. C., Kadoo N. Y., Ghorpade P. B., Jana M. M. & Gupta V. S. (2012). Development of genomic simple sequence repeat markers for linseed using next-generation sequencing technology. *Molecular Breeding* 30, 597-606.

Kaleb S., Falace A. & Woelkerling W. (2012). *Phymatolithon lamii* (Hapalidiaceae, Corallinales, Rhodophyta): a first report for the Mediterranean Sea. *Botanica Marina* 55, 377–385.

Kalia R. K., Rai M. K., Kalia S., Singh R. & Dhawan A. K. (2011). Microsatellite markers: an overview of the recent progress in plants. *Euphytica* 177, 309-334.

Kamenos N. A., Moore P. G. & Hall Spencer J. M. (2004a). Attachment of the juvenile queen scallop (*Aequipecten opercularis* (L.)) to maerl in mesocosm conditions; juvenile habitat selection. *Journal of Experimental Marine Biology & Ecology* 306, 139-155.

Kamenos N. A., Moore P. G. & Hall Spencer J. M. (2004b). Maerl grounds provide both refuge and high growth potential for juvenile queen scallops (*Aequipecten opercularis* L.). *Journal of Experimental Marine Biology & Ecology* 313, 241-254.

Kamenos N. A., Moore P. G. & Hall Spencer J. M. (2004c). Nursery-area function of maerl grounds for juvenile queen scallops *Aequipecten opercularis* and other invertebrates. *Marine Ecology Progress Series* 274, 183-189.

Kamenos N. A., Moore P. G. & Hall Spencer J. M. (2004d). Small-scale distribution of juvenile gadoids in shallow inshore waters; what role does maerl play? *ICES Journal of Marine Science* 61, 422-429.

Kass R. E. & Raftery A. E. (1995). Bayes Factors. *Journal of the American Statistical Association* 90, 773-795.

Khodami S., Martinez Arbizu P., Stöhr S. & Laakmann S. (2014). Molecular species delimitation of Icelandic brittle stars (Ophiuroidea). *Polish Polar Research* 35, 243-260.

Kim M. S., Koh Y. H. & Cho G. Y. (2013). Species delimitation of the genus *Champia* (Rhodymeniales, Rhodophyta) from Korea using DNA barcoding. *Journal of Ecology & Environment* 36, 449-463.

Kimura M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide-sequences. *Journal of Molecular Evolution* 16, 111-120.

Kjellman F. R. (1883). Norra ishafvets algflora. In: *Vega-expeditionens Vetenskapliga Iakttagelser* (ed. Nordenskiöld, A. E.), pp. 1-431. F. & G. Beijers Förlag, Stockholm, Sweden.

Kollars N. M., Krueger-Hadfield S. A., Byers J. E., Greig T. W., Strand A. E., Weinberger F. & Sotka E. E. (2015). Development and characterization of microsatellite loci for the haploid-diploid red seaweed *Gracilaria vermiculophylla*. *PeerJ* 3, e1159 (p. 11).

Konar B., Riosmena-Rodriguez R. & Iken K. (2006). Rhodolith bed: a newly discovered habitat in the North Pacific Ocean. *Botanica Marina* 49, 355-359.

Kong F., Mao Y., Yang H., Qu H., Yan X. & Wang L. (2009). Genetic analysis of *Porphyra yezoensis* using microsatellite markers. *Plant Molecular Biology Reporter* 27, 496-502.

Kostamo K., Korpelainen H. & Olsson S. (2012). Comparative study on the population genetics of the red algae *Furcellaria lumbricalis* occupying different salinity conditions. *Marine Biology* 159, 561-571.

Králová-Hromadová I., Minárik G., Bazsalovicsová E., Mikulíček P., Oravcová A., Pálková L. & Hanzelová V. (2015). Development of microsatellite markers in *Caryophyllaeus laticeps* (Cestoda: Caryophyllidea), monozoic fish tapeworm, using next-generation sequencing approach. *Parasitology Research* 114, 721-726.

Krueger-Hadfield S. A., Collen J., Daguin-Thiebaut C. & Valero M. (2011). Genetic population structure and mating system in *Chondrus crispus* (Rhodophyta). *Journal of Phycology* 47, 440-450.

Krueger-Hadfield S. A., Roze D., Mauger S. & Valero M. (2013). Intergametophytic selfing and microgeographic genetic structure shape populations of the intertidal red seaweed *Chondrus crispus*. *Molecular Ecology* 22, 3242-3260.

Kubatko L. S., Carstens B. C. & Knowles L. L. (2009). STEM: species tree estimation using maximum likelihood for gene trees under coalescence. *Bioinformatics* 25, 971-973.

Kucera H. & Saunders G. W. (2012). A survey of Bangiales (Rhodophyta) based on multiple molecular markers reveals cryptic diversity. *Journal of Phycology* 48, 869-882.

Kumar P., Gupta V. K., Misra A. K., Modi D. R. & Pandey B. K. (2009). Potential of molecular markers in plant biotechnology. *Plant Omics Journal* 2, 141-162.

Lachance J. & Tishkoff S. A. (2013). SNP ascertainment bias in population genetic analyses: why it is important, and how to correct it. *Bioessays* 35, 780-786.

Lartillot N. & Philippe H. (2006). Computing Bayes Factors using thermodynamic integration. *Systematic Biology* 55, 195-207.

Le Gall L. & Saunders G. W. (2007). A nuclear phylogeny of the Florideophyceae (Rhodophyta) inferred from combined EF2, small subunit and large subunit ribosomal DNA: establishing the new red subalgal class Corallinophycidae. *Molecular Phylogenetics & Evolution* 43, 1118-1130.

Le Gall L. & Saunders G. W. (2010a). Establishment of a DNA-barcode library for the Nemaliales (Rhodophyta) from Canada and France uncovers overlooked diversity in the species

Nemalion helminthoides (Velley) Batters. *Cryptogamie, Algologie* 31, 403-421.

Le Gall L. & Saunders G. W. (2010b). DNA barcoding is a powerful tool to uncover algal diversity: a case study of the Phyllophoraceae (Gigartinales, Rhodophyta) in the Canadian flora. *Journal of Phycology* 46, 374-389.

Lecocq T., Dellicour S., Michez D., Dehon M., Dewulf A., de Meulemeester T., Brasero N., Valterová I., Rasplus J.-Y. & Rasmont P. (2015). Methods for species delimitation in bumblebees (Hymenoptera, Apidae, *Bombus*): towards an integrative approach. *Zoologica Scripta* 44, 281-297.

Leliaert F., Verbruggen H., Vanormelingen P., Steen F., López-Bautista J. M., Zuccarello G. C. & De Clerck O. (2014). DNA-based species delimitation in algae. *European Journal of Phycology* 49, 179-196.

Lemay M. A. & Russello M. A. (2015). Genetic evidence for ecological divergence in kokanee salmon. *Molecular Ecology* 24, 798-811.

Lemoine M. (1931). Les algues mélobésiées de la région de Saint-Servan. *Bulletin du Laboratoire Maritime du Museum d'Histoire Naturelle de Saint-Servan* 7, 1-20.

Lemoine M. P. (1910). Répartition et mode de vie du Maërl (*Lithothamnium calcareum*) aux environs de Concarneau (Finistère). *Annales de Institut Océanographique. Fondation Albert I^{er}, Prince de Monaco* 1, 1-28.

Lemoine M. P. (1913). Mélobésiées de l'ouest de l'Irlande (Clew Bay). *Nouvelle Archives du Muséum National d'Histoire Naturelle de Paris* 5, 121-145.

Li Y.-C., Korol A. B., Fahima T., Beiles A. & Nevo E. (2002). Microsatellites: genomic distribution, putative functions and mutational mechanisms: a review. *Molecular Ecology* 11, 2453-2465.

Librado P. & Rozas J. (2009). DnaSP v.5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25, 1451-1452.

Liu B.-Q., Zeng Q.-G., Luo Q.-J., Wang Y.-J. & Li S.-H. (2005). Isolation of microsatellite loci from dbEST of algae *Porphyra yezoensis* and primer amplification of interspecies transfer. *Oceanologia et Limnologia Sinica* 36, 248-254.

Liu L. (2008). BEST: Bayesian estimation of species trees under the coalescent model. *Bioinformatics* 24, 2542-2543.

Lopes J. S. & Beaumont M. A. (2010). ABC: a useful Bayesian tool for the analysis of population data. *Infection, Genetics & Evolution* 10, 825-832.

López-Uribe M. M., Santiago C. K., Bogdanowicz S. M. & Danforth B. N. (2013). Discovery and characterization of microsatellites for the solitary bee *Colletes inaequalis* using Sanger and 454 pyrosequencing. *Apidologie* 44, 163-172.

Luo H., Mörchen M., Engel C. R., Destombe C., Epplen J. T., Epplen C., Saumitou-Laprade P. & Valero M. (1999). Characterization of microsatellite markers in the red alga *Gracilaria gracilis*. *Molecular Ecology* 8, 700-702.

Lyra G. d. M., Costa E. d. S., de Jesus P. B., de Matos J. C. G., Caires T. A., Oliveira M. C., Oliveira E. C., Xi Z., Nunes J. M. d. C. & Davis C. C. (2015). Phylogeny of Gracilariaceae (Rhodophyta): evidence from plastid and mitochondrial nucleotide sequences. *Journal of Phycology* 51, 356-366.

Maggs C. A. (1983). *A phenological study of two maerl beds in Galway Bay, Ireland*. PhD thesis, National University of Ireland, Galway.

Maggs C. A. (1988). Intraspecific life history variability in the Florideophycidae (Rhodophyta). *Botanica Marina* 31, 465-490.

Malausa T., Gilles A., Meglécz E., Blanquart H., Duthoy S., Costedoat C., Dubut V., Pech N., Castagnone-Sereno P., Délye C., Feau N., Frey P., Gauthier P., Guillemaud T., Hazard L., Le Corre V., Lung-Escarmant B., Malé P.-J. G., Ferreira S. & Martin J.-F. (2011). High-throughput microsatellite isolation through 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries. *Molecular Ecology Resources* 11, 638-644.

Mallet J. (1995). A species definition for the modern synthesis. *Trends in Ecology & Evolution* 10, 294-299.

Mamoozadeh N. R. & Freshwater D. W. (2012). *Polysiphonia sensu lato* (Ceramiales, Florideophyceae) species of Caribbean Panama including *Polysiphonia lobophoralis* sp. nov. and *Polysiphonia nuda* sp. nov. *Botanica Marina* 55, 317-347.

Manghisi A., Le Gall L., Ribera M. A., Bonillo C., Gargiulo G. M. & Morabito M. (2014). The Mediterranean endemic new genus *Felicinia* (Halymeniales, Rhodophyta) recognized by a morphological and phylogenetic integrative approach. *Cryptogamie, Algologie* 35, 221-243.

Manghisi A., Morabito M., Bertuccio C., Le Gall L., Couloux A., Cruaud C. & Genovese G. (2010). Is routine DNA barcoding an efficient tool to reveal introductions of alien macroalgae? A case study of *Agardhiella subulata* (Solieriaceae, Rhodophyta) in Cape Peloro lagoon (Sicily, Italy). *Cryptogamie, Algologie* 31, 423-433.

Martínez E. A., Cárdenas L., Figueroa C., Vidal R. U. & Billot C. (2005). Microsatellites of *Laminaria digitata* tested in *Lessonia nigrescens*:

evaluation and improvement of cross amplification between kelps of two different families. *Journal of Applied Phycology* 17, 245-253.

Mayr E. (1942). *Systematics and the Origin of Species from the Viewpoint of a Zoologist.* Columbia University Press, New York, USA.

Mayr E. (1996). What is a species, and what is not? *Philosophy of Science* 63, 262-277.

McCoy S. J. & Kamenos N. A. (2015). Coralline algae (Rhodophyta) in a changing world: integrating ecological, physiological, and geochemical responses to global change. *Journal of Phycology* 51, 6-24.

McNeely J. A., Miller K. R., Reid W. V., Mittermeier R. A. & Werner T. B. (1990). Conserving the world's biological diversity, p. 193. IUCN, Gland, Switzerland; WRI, CI, WWF-US, and The World Bank, Washington D.C., USA.

Meffe G. K. & Carroll C. R. (1994). *Principles of Conservation Biology.* Sinauer Associates Inc., Massachusetts, USA.

Meglécz E., Costedoat C., Dubut V., Gilles A., Malausa T., Pech N. & Martin J.-F. (2010). QDD: a user-friendly program to select microsatellite markers and design primers from large sequencing projects. *Bioinformatics* 26, 403-404.

Mendoza M. L. & Cabioch J. (1998). Étude comparée de la reproduction de *Phymatolithon calcareum* (Pallas) Adey & McKibbin et *Lithothamnion corallioides* (P. & H. Crouan) P. & H. Crouan (Corallinales, Rhodophyta), et reconsidérations sur le définition des genres. *Canadian Journal of Botany* 76, 1433-1445.

Metzker M. L. (2010). Sequencing technologies—the next generation. *Nature Reviews, Genetics* 11, 31-46.

Meyer C. P. & Paulay G. (2005). DNA barcoding: error rates based on comprehensive sampling. *PLoS Biology* 3, e422 (p. 10).

Milstein D. & Saunders G. W. (2012). DNA barcoding of Canadian Ahnfeltiales (Rhodophyta) reveals a new species - *Ahnfeltia borealis* sp. nov. *Phycologia* 51, 247-259.

Millar M. A., Byrne M. & Coates D. J. (2010). The maintenance of disparate levels of clonality, genetic diversity and genetic differentiation in disjunct subspecies of the rare *Banksia ionthocarpa*. *Molecular Ecology* 19, 4217-4227.

Minárik G., Bazsalovicsová E., Zvijáková Ľ., Štefka J., Pálková L. & Královál-Hromadová I. (2014). Development and characterization of multiplex panels of polymorphic microsatellite loci in giant liver fluke *Fascioloides magna* (Trematoda: Fasciolidae), using next-generation sequencing approach. *Molecular & Biochemical Parasitology* 195, 30-33.

Mishler B. D. (1985). The morphological, developmental, and phylogenetic basis of species concepts in Bryophytes. *The Bryologist* 88, 207-214.

Monaghan M. T., Wild R., Elliot M., Fujisawa T., Balke M., Inward D. J. G., Lees D. C., Ranaivosolo R., Eggleton P., Barraclough T. G. & Vogler A. P. (2009). Accelerated species inventory on Madagascar using coalescent-based models of species delineation. *Systematic Biology* 58, 298-311.

Moritz C. & Cicero C. (2004). DNA Barcoding: promise and pitfalls. *PLoS Biology* 2, e354 (p. 3).

Nauer F., Guimarães N., Cassano V., Yokoya N. S. & Oliveira M. C. (2014). *Hypnea* species (Gigartinales, Rhodophyta) from the southeastern coast of Brazil based on molecular studies complemented with morphological analyses, including descriptions of *Hypnea edeniana* sp. nov. and *H. flava* sp. nov. *European Journal of Phycology* 49, 550-575.

Necchi O. Jr., Garcia Fo. A. S., Salomaki E. D., West J. A., Aboal M. & Vis M. L. (2013). Global sampling reveals low genetic diversity within *Compsopogon* (Compsopogonales, Rhodophyta). *European Journal of Phycology* 48, 152-162.

Nelson W. A. (2009). Calcified macroalgae - critical to coastal ecosystems and vulnerable to change: a review. *Marine & Freshwater Research* 60, 787-801.

Nelson W. A., Sutherland J. E., Farr T. J., Hart D. R., Neill K. F., Kim H. J. & Yoon H. S. (2015). Multi-gene phylogenetic analyses of New Zealand coralline algae: *Corallinapetra novaezelandiae* gen. et sp. nov. and recognition of the Hapalidiales ord. nov. *Journal of Phycology* 51, 454-468.

Newton M. A. & Raftery A. E. (1994). Approximate bayesian inference with the weighted likelihood bootstrap. *Journal of the Royal Statistical Society. Series B (Methodological)* 56, 3-48.

Nielsen R. & Signorovitch J. (2003). Correcting for ascertainment biases when analyzing SNP data: applications to the estimation of linkage disequilibrium. *Theoretical Population Biology* 63, 245-255.

Noisette F., Duong G., Six C., Davoult D. & Martin S. (2013a). Effects of elevated pCO_2 on the metabolism of a temperate rhodolith *Lithothamnion corallioides* grown under different temperatures. *Journal of Phycology* 49, 746-757.

Noisette F., Egilsdottir H., Davoult D. & Martin S. (2013b). Physiological responses of three

temperate coralline algae from contrasting habitats to near-future ocean acidification. *Journal of Experimental Marine Biology & Ecology* 448, 179-187.

O'Doherty D. C. & Sherwood A. R. (2007). Genetic population structure of the Hawaiian alien invasive seaweed *Acanthophora spicifera* (Rhodophyta) as revealed by DNA sequencing and ISSR analyses. *Pacific Science* 61, 223-233.

O'Meara B. C. (2010). New heuristic methods for joint species delimitation and species tree inference. *Systematic Biology* 59, 59-73.

O'Meara B. C., Ané C., Sanderson M. J. & Wainwright P. C. (2006). Testing for different rates of continuous trait evolution using likelihood. *Evolution* 60, 922-933.

Oliveira E. J., Padúa J. G., Zucchi M. I., Vencovsky R. & Vieira M. L. C. (2006). Origin, evolution and genome distribution of microsatellites. *Genetics and Molecular Biology* 29, 294-307.

Olsen J. L., Sadowski G., Stam W. T., Veldsink J. H. & Jones K. (2002). Characterization of microsatellite loci in the marine seaweed *Ascophyllum nodosum* (Phaeophyceae; Fucales). *Molecular Ecology Notes* 2, 33-34.

Oppliger V. L., von Dassow P., Bouchemousse S., Robuchon M., Valero M., Correa J. A., Mauger S. & Destombe C. (2014). Alteration of sexual reproduction and genetic diversity in the kelp species *Laminaria digitata* at the southern limit of its range. *PLoS ONE* 9, e102518 (p. 11).

Padial J. M., Miralles A., De la Riva I. & Vences M. (2010). The integrative future of taxonomy. *Frontiers in Zoology* 7, 16 (p. 14).

Pan J. J. & Price J. S. (2001). Fitness and evolution in clonal plants: the impact of clonal growth. *Evolutionary Ecology* 15, 583-600.

Pardo C., Lopez L., Peña V., Hernandez-Kantun J., Le Gall L., Barbara I. & Barreiro R. (2012). A DNA-barcoding study fosters our understanding of the distribution and magnitude of maërl biodiversity in the OSPAR area. In: *Book of Abstracts of the IV International Rhodolith Workshop, September 17-21, 2009. Facultad de Ciencias de la Universidad de Granada* (eds. Aguirre J., Rösler A. & Braga J. C.), pp. 55-55, Granada, Spain.

Pardo C., López L., Peña V., Hernández-Kantún J., Le Gall L., Bárbara I. & Barreiro R. (2014a). A multilocus species delimitation reveals a striking number of species of coralline algae forming maerl in the OSPAR maritime area. *PLoS ONE* 9, e104073 (p. 12).

Pardo C., Peña V., Bárbara I., Valero M. & Barreiro R. (2014b). Development and multiplexing of the first microsatellite markers in a coralline red alga (*Phymatolithon calcareum*, Rhodophyta). *Phycologia* 53, 474-479.

Pardo C., Peña V., Barreiro R. & Bárbara I. (2015a). A molecular and morphological study of *Corallina sensu lato* (Corallinales, Rhodophyta) in the Atlantic Iberian Peninsula. *Cryptogamie, Algologie* 36, 31-54.

Pardo C., Peña V., Bárbara I., Valero M. & Barreiro R. (2015b). Poster: A pioneer microsatellite study of clonality in a major maerl-forming species *Phymatolithon calcareum* (Rhodophyta) in Atlantic Europe. *European Journal of Phycology* 50 (S1), 128-128.

Park S. D. E. (2001). *Trypanotolerance in West African cattle and the population genetic effects of selection.* PhD thesis, University of Dublin, Ireland.

Payo D. A., Leliaert F., Verbruggen H., D'Hondt S., Calumpong H. P. & De Clerck O. (2013). Extensive cryptic species diversity and fine-scale endemism in the marine red alga *Portieria* in the Philippines. *Proceedings of the Royal Society B: Biological Sciences* 280, 20122660 (p. 8).

Pearson E. A. & Murray S. N. (1997). Patterns of reproduction, genetic diversity, and genetic differentiation in California populations of the geniculate coralline alga *Lithothrix aspergillum* (Rhodophyta). *Journal of Phycology* 33, 753–763.

Pearson R. G. & Dawson T. P. (2003). Predicting the impacts of climate change on the distribution of species: are bioclimate envelope models useful? *Global Ecology & Biogeography* 12, 361-371.

Peña Freire V. (2010). *Estudio ficológico de los fondos de maërl y cascajo en el noroeste de la Península Ibérica*. PhD thesis, Universidade da Coruña, Spain.

Peña V., Adey W. H., Riosmena-Rodríguez R., Jung M.-Y., Afonso-Carrillo J., Choi H. G. & Bárbara I. (2011). *Mesophyllum sphaericum* sp. nov. (Corallinales, Rhodophyta): a new maërl-forming species from the northeast Atlantic. *Journal of Phycology* 47, 911-927.

Peña V. & Bárbara I. (2004). Diferenciación morfológica y anatómica entre *Lithothamnion corallioides* y *Phymatolithon calcareum* (Corallinales, Rhodophyta) en dos bancos de maërl de la Ría de Arousa (N. O. Península Ibérica). *Anales de Biología* 26, 21-27.

Peña V. & Bárbara I. (2008a). Maërl community in the north-western Iberian Peninsula: a review of floristic studies and long-term changes. *Aquatic Conservation: Marine & Freshwater Ecosystems* 18, 339-366.

Peña V. & Bárbara I. (2008b). Biological importance of an Atlantic European maërl bed off Benencia Island (northwest Iberian Peninsula). *Botanica Marina* 51, 493-505.

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., Berecibar E. & Santos R. (2009). Present distribution of maërl beds in the Atlantic Iberian Peninsula. In: *Abstract book of the 6th Regional Symposium of the International Fossil Algae Associaton, Milano, July 1-5, 2009. Annali dell'Università di Ferrera, Sez. Museologia Scientifica e Naturalistica, volume speciale* (eds. Basso D., Caragnano A., Bracchi V. & Benzoni F.), pp. 46-47, Ferrara, Italy.

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, 533-551.

Peña V., De Clerck O., Afonso-Carrillo J., Ballesteros E., Bárbara I., Barreiro R. & Le Gall L. (2015a). An integrative systematic approach to species diversity and distribution in the genus *Mesophyllum* (Corallinales, Rhodophyta) in Atlantic and Mediterranean Europe. *European Journal of Phycology* 50, 20-36.

Peña V., Hernández-Kantún J. 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.

Peña V., Pardo C., López L., Carro B., Hernández-Kantún J., Adey W. H., Bárbara I., Barreiro R. & Le Gall L. (2015b). *Phymatolithon lusitanicum* sp. nov. (Hapalidiales, Rhodophyta): the third most abundant maerl-forming species in the Atlantic Iberian Peninsula. *Cryptogamie Algologie* 36, 429-459.

Perestenko L. P. (1994). *Krasnye vodorosli dal'nevostochnykh more Rossii.* Rossiiskaia Akademiia Nauk, Botanichesk Institut im. V.L. Komarova, St. Petersburg, Russian Federation.

Pons J., Barraclough T. G., Gomez-Zurita J., Cardoso A., Duran D. P., Hazell S., Kamoun S., Sumlin W. D. & Vogler A. P. (2006). Sequence-based species delimitation for the DNA taxonomy of undescribed insects. *Systematic Biology* 55, 595-609.

Powell J. R. (2012). Accounting for uncertainty in species delineation during the analysis of environmental DNA sequence data. *Methods in Ecology & Evolution* 3, 1-11.

Presting G. G. (2006). Identification of conserved regions in the plastid genome: implications for DNA barcoding and biological function. *Canadian Journal of Botany* 84, 1434-1443.

Preuss M. & Zuccarello G. C. (2014). What's in a name? Monophyly of genera in the red algae: *Rhodophyllis parasitica* sp. nov. (Gigartinales, Rhodophyta); a new red algal parasite from New Zealand. *Algae* 29, 279-288.

Provan J., Glendinning K., Kelly R. & Maggs C. A. (2013). Levels and patterns of population genetic diversity in the red seaweed *Chondrus crispus* (Florideophyceae): a direct comparison of single nucleotide polymorphisms and microsatellites. *Biological Journal of the Linnean Society* 108, 251-262.

Provan J. & Maggs C. A. (2012). Unique genetic variation at a species' rear edge is under threat from global climate change. *Proceedings of the Royal Society B: Biological Sciences* 279, 39-47.

Provan J., Powell W. & Hollingsworth P. M. (2001). Chloroplast microsatellites: new tools for studies in plant ecology and evolution. *Trends in Ecology & Evolution* 16, 142-147.

Puillandre N., Lambert A., Brouillet S. & Achaz G. (2012a). ABGD, Automatic Barcode Gap Discovery for primary species delimitation. *Molecular Ecology* 21, 1864-1877.

Puillandre N., Modica M. V., Zhang Y., Sirovich L., Boisselier M. C., Cruaud C., Holford M. & Samadi S. (2012b). Large-scale species delimitation method for hyperdiverse groups. *Molecular Ecology* 21, 2671-2691.

Putman A. I. & Carbone I. (2014). Challenges in analysis and interpretation of microsatellite data for population genetic studies. *Ecology & Evolution* 4, 4399-4428.

Rajendrakumar P., Biswal A. K., Balachandran S. M., Srinivasarao K. & Sundaram R. M. (2007). Simple sequence repeats in organellar genomes of rice: frequency and distribution in genic and intergenic regions. *Bioinformatics* 23, 1-4.

Rannala B. & Yang Z. (2003). Bayes estimation of species divergence times and ancestral population sizes using DNA sequences from multiple loci. *Genetics* 164, 1645-1656.

Ratnasingham S. & Hebert P. D. N. (2007). BOLD: The Barcode of Life Data System (www.barcodinglife.org). *Molecular Ecology Notes* 7, 355-364.

Reed D. & Frankham R. (2003). Correlation between fitness and genetic diversity. *Conservation Biology* 17, 230-237.

Reid N. M. & Carstens B. C. (2012). Phylogenetic estimation error can decrease the accuracy of species delimitation: a Bayesian implementation of the general mixed Yule-coalescent model. *BMC Evolutionary Biology* 12, 196 (p. 11).

Rix L. N., Burdett H. L. & Kamenos N. A. (2012). Irradiance-mediated dimethylsulphoniopropionate (DMSP) responses of red coralline algae. *Estuarine, Coastal & Shelf Science* 96, 268-272.

Robba L., Russell S. J., Barker G. L. & Brodie J. (2006). Assessing the use of the mitochondrial *cox*1 marker for use in DNA barcoding of red algae (Rhodophyta). *American Journal of Botany* 93, 1101-1108.

Ronquist F. & Huelsenbeck J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19, 1572-1574.

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.

Rosenvinge M. L. K. (1894). Les algues marine du Groenland. *Annales des Sciences Naturelles* 7, 53-164.

Roy V., Constantino R., Chassany V., Giusti-Miller S., Diouf M., Mora P. & Harry M. (2014). Species delimitation and phylogeny in the genus *Nasutitermes* (Termitidae: Nasutitermitinae) in French Guiana. *Molecular Ecology* 23, 902-920.

Rozen S. & Skaletsky H. (2000). Primer3 on the WWW for general users and for biologist programmers. *Methods in Molecular Biology* 132, 365-386.

Rueness J. (2010). DNA barcoding of select freshwater and marine red algae (Rhodophyta). *Cryptogamie, Algologie* 31, 377-386.

Salomaki E. D., Kwandrans J., Eloranta P. & Vis M. L. (2014). Molecular and morphological evidence for *Sheathia* gen. nov. (Batrachospermales, Rhodophyta) and three new species. *Journal of Phycology* 50, 526-542.

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.

Saunders G. W. (2008). A DNA barcode examination of the red algal family Dumontiaceae in Canadian waters reveals substantial cryptic species diversity. 1. The foliose *Dilsea-Neodilsea* complex and *Weeksia*. *Botany* 86, 773-789.

Saunders G. W. (2009). Routine DNA barcoding of Canadian Gracilariales (Rhodophyta) reveals the invasive species *Gracilaria vermiculophylla* in British Columbia. *Molecular Ecology Resources* 9 (S1), 140-150.

Saunders G. W. & McDevit D. C. (2012a). Acquiring DNA sequence data from dried archival red algae (Florideophyceae) for the purpose of applying available names to contemporary genetic species: a critical assessment. *Botany* 90, 191-203.

Saunders G. W. & McDevit D. C. (2012b). Methods for DNA barcoding photosynthetic protists emphasizing the macroalgae and diatoms. *Methods in Molecular Biology* 858, 207-222.

Saunders G. W. & McDevit D. C. (2013). DNA barcoding unmasks overlooked diversity improving knowledge on the composition and origins of the Churchill algal flora. *BMC Ecology* 13, 9 (p. 23).

Saunders G. W. & McDonald B. (2010). DNA barcoding reveals multiple overlooked Australian species of the red algal order Rhodymeniales (Florideophyceae), with resurrection of *Halopeltis* J. Agardh and description of *Pseudohalopeltis* gen. nov. *Botany* 88, 639-667.

Sauriau P.-G., Curti C., Jourde J., Aubert F., Cajeri P., Lavesque N., Dubois S., Lepareur F., Gouesbier C., Sauriau F., Sauriau M., Latry L., Leguay D., Robert S., Pineau P. & Geairon P. (2012). Le maerl algues Corallinacées marines dans les Pertuis Charentais. *Annales de la Société des Sciences Naturelles de Charente-Maritime* 10, 281-300.

Schaeffer T. N., Smith G. J., Foster M. S. & DeTomaso A. (2002). Genetic differences between two growth-forms of *Lithophyllum margaritae* (Rhodophyta) in Baja California Sur, Mexico. *Journal of Phycology* 38, 1090-1098.

Schlick-Steiner B. C., Steiner F. M., Seifert B., Stauffer C., Christian E. & Crozier R. H. (2010). Integrative taxonomy: a multisource approach to exploring biodiversity. *Annual Review of Entomology* 55, 421-438.

Schlötterer C. (2004). The evolution of molecular markers - just a matter of fashion? *Nature Reviews, Genetics* 5, 63-69.

Schoebel C. N., Brodbeck S., Buehler D., Cornejo C., Gajurel J., Hartikainen H., Keller D., Leys M., Říčanová Š., Segelbacher G., Werth S. & Csencsics D. (2013). Lessons learned from microsatellite development for nonmodel organisms using 454 pyrosequencing. *Journal of Evolutionary Biology* 26, 600-611.

Selkoe K. A. & Toonen R. J. (2006). Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. *Ecology Letters* 9, 615-629.

Sherwood A. R., Kurihara A., Conklin K. Y., Sauvage T. & Presting G. G. (2010a). The Hawaiian Rhodophyta biodiversity survey (2006-2010): a summary of principal findings. *BMC Plant Biology* 10, 258-287.

Sherwood A. R., Sauvage T., Kurihara A., Conklin K. Y. & Presting G. G. (2010b). A comparative analysis

of COI, LSU and UPA marker data for the Hawaiian florideophyte Rhodophyta: implications for DNA barcoding of red algae. *Cryptogamie, Algologie* 31, 451-465.

Sherwood A. R., Vis M. L., Entwisle T. J., Necchi O. Jr. & Presting G. G. (2008). Contrasting intra *versus* interspecies DNA sequence variation for representatives of the Batrachospermales (Rhodophyta): insights from a DNA barcoding approach. *Phycological Research* 56, 269-279.

Shi Y., Yang G., Liu Y., Liao M., Li X. & Cong Y. (2007). Development of 18 polymorphic microsatellite DNA markers of *Laminaria japonica* (Phaeophyceae). *Molecular Ecology Notes* 7, 620-622.

Silvertown J. (2008). The evolutionary maintenance of sexual reproduction: evidence from the ecological distribution of asexual reproduction in clonal plants. *International Journal of Plant Sciences* 169, 157-168.

Sissini M. N., Oliveira M. C., Gabrielson P. W., Robinson N. M., Okolodkov Y. B., Riosmena Rodríguez R. & Horta P. A. (2014). *Mesophyllum erubescens* (Corallinales, Rhodophyta) - so many species in one epithet. *Phytotaxa* 190, 299-319.

Sites J. W. & Marshall J. C. (2003). Delimiting species: a Renaissance issue in systematic biology. *Trends in Ecology & Evolution* 18, 462-470.

Song S.-L., Lim P.-E., Phang S.-M., Lee W.-W., Hong D. D. & Prathep A. (2014). Development of chloroplast simple sequence repeats (cpSSRs) for the intraspecific study of *Gracilaria tenuistipitata* (Gracilariales, Rhodophyta) from different populations. *BMC Research Notes* 7, 77-85.

Song S.-L., Lim P.-E., Phang S.-M., Lee W.-W., Lewmanomont K., Largo D. B. & Han N. A. (2013). Microsatellite markers from expressed sequence tags (ESTs) of seaweeds in differentiating various *Gracilaria* species. *Journal of Applied Phycology* 25, 839-846.

Soulé M. E. (1985). What is Conservation Biology? *BioScience* 35, 727-734.

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 & Ecology* 194, 201-212.

Steneck R. S. (1986). The ecology of coralline algal crusts: convergent patterns and adaptative strategies. *Annual Review of Ecology & Systematics* 17, 273-303.

Strömfelt H. F. G. (1886). *Om Algvegetationen vid Islands Kuster.* Akademisk Afhandling, Göteborg, Sweden.

Sun J., Liu T., Guo B., Jin D., Weng M., Feng Y., Xu P., Duan D. & Wang B. (2006). Development of SSR primers from EST sequences and their application in germplasm identification of *Porphyra* lines (Rhodophyta). *European Journal of Phycology* 41, 329-336.

Suneson S. (1937). Studien über die entwicklungsgeschichte der Corallinaceen. *Lunds Universitets Ärsskrift* 33, 1-101.

Suneson S. (1943). The structure, life history and taxonomy of the Swedish Corallinaceae. *Lunds Universitets Ärsskrift* 39, 1-66.

Suneson S. (1950). The cytology of the bispore formation of two species of *Lithophyllum* (*L. litorale* and *L. corallinae*) and the significance of the bispores in the Corallinaceae. *Botaniska Notiser* 4, 429-450.

Suneson S. (1958). *Lithothamnion calcareum* vid svenska västkusten. *Botaniska Notiser* 111, 195-199.

Suneson S. (1982). The culture of bisporangial plants of *Dermatolithon litorale* (Suneson) Hamel et Lemoine (Rhodophyta, Corallinaceae). *British Phycological Journal* 17, 107-116.

Takayama K., López S. P., Köenig C., Kohl G., Novak J. & Stuessy T. F. (2011). A simple and cost-effective approach for microsatellite isolation in non-model plant species using small-scale 454 pyrosequencing. *Taxon* 60, 1442-1449.

Tamura K., Stecher G., Peterson D., Filipski A. & Kumar S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology & Evolution* 30, 2725-2729.

Tan J., Lim P.-E., Phang S.-M., Hong D. D., Sunarpi H. & Hurtado A. Q. (2012). Assessment of four molecular markers as potential DNA barcodes for red algae *Kappaphycus* Doty and *Eucheuma* J. Agardh (Solieriaceae, Rhodophyta). *PLoS ONE* 7, e52905 (p. 15).

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.

Thiers B. (2015). *Index Herbariorum: A Global Directory of Public Herbaria and Associated Staff.* http://sweetgum.nybg.org/ih/. Revised on 10 July 2015, New York Botanical Garden's Virtual Herbarium, USA.

Travis S. E. & Hester M. W. (2005). A space-for-time substitution reveals the long-term decline in genotypic diversity of a widespread salt marsh plant, *Spartina alterniflora*, over a span of 1500 years. *Journal of Ecology* 93, 417-430.

Unger F. (1858). Beiträge zur näheren Kenntniss des Leithakalkes, namentlich der vegetabilischen Einschlüsse und der Bildungsgeschichte

Desselben. *Denkschriften der Kaiserlichen Akademie der Wissenschaften in Wien, Mathematisch-Naturwissenschaftliche Klasse* 14, 13-35.

Vallejo-Marín M., Dorken M. E. & Barrett S. C. H. (2010). The ecological and evolutionary consequences of clonality for plant mating. *Annual Review of Ecology, Evolution & Systematics* 41, 193-213.

Van Valen L. (1976). Ecological species, multispecies, and oaks. *Taxon* 25, 233-239.

Vergés A., Gey D., Utgé J., Cruaud C. & Le Gall L. (2014). Recognition of a new species of *Kallymenia* (Gigartinales, Rhodophyta) from Croatia (Mediterranean Sea) based on morphology and DNA barcode. *European Journal of Phycology* 49, 332-344.

Vieira C., D'hondt S., De Clerck O. & Payri C. E. (2014). Toward an inordinate fondness for stars, beetles and *Lobophora*? Species diversity of the genus *Lobophora* (Dictyotales, Phaeophyceae) in New Caledonia. *Journal of Phycology* 50, 1101-1119.

Vis M. L., Necchi O. Jr., Chiasson W. B. & Entwisle T. J. (2012). Molecular phylogeny of the genus *Kumanoa* (Batrachospermales, Rhodophyta). *Journal of Phycology* 48, 750-758.

Wachter G. A., Muster C., Arthofer W., Raspotnig G., Föttinger P., Komposch C., Steiner F. M. & Schlick-Steiner B. C. (2015). Taking the discovery approach in integrative taxonomy: decrypting a complex of narrow-endemic Alpine harvestmen (Opiliones: Phalangiidae: *Megabunus*). *Molecular Ecology* 24, 863-889.

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.

Wan Q.-H., Wu H., Fujihara T. & Fang S.-G. (2004). Which genetic marker for which conservation genetics issue? *Electrophoresis* 25, 2165–2176.

Wang J., Peng C., Liu Z., Tang Z. & Yang G. (2013). Isolation and characterization of microsatellites of *Grateloupia filicina*. *Conservation Genetics Resources* 5, 763-766.

Wattier R., Dallas J. F., Destombe C., Saumitou-Laprade P. & Valero M. (1997). Single locus microsatellites in Gracilariales (Rhodophyta): high level of genetic variability within *Gracilaria gracilis* and conservation in related species. *Journal of Phycology* 33, 868-880.

West J. A., Scott J. L., West K. A., Karsten U., Clayden S. L. & Saunders G. W. (2008). *Rhodachlya madagascarensis* gen. et sp. nov.: a distinct acrochaetioid represents a new order and family (Rhodachlyales ord. nov., Rhodachlyaceae fam. nov.) of the Florideophyceae (Rhodophyta). *Phycologia* 47, 203-212.

Wheeler Q. D. (2008). *The New Taxonomy.* CRC Press, Boca Raton, Florida, USA.

Whitmer A. C. (2002). Microsatellite markers for the intertidal kelp *Postelsia palmaeformis* (Heterokontophyta; Laminariales). *Molecular Ecology Notes* 2, 469-471.

Wiemers M. & Fiedler K. (2007). Does the DNA barcoding gap exist? - a case study in blue butterflies (Lepidoptera: Lycaenidae). *Frontiers in Zoology* 4, 8 (p. 16).

Wiens J. J. & Penkrot T. A. (2002). Delimiting species using DNA and morphological variation and discordant species limits in spiny lizards (*Sceloporus*). *Systematic Biology* 51, 69-91.

Wilson S., Blake C., Berges J. A. & Maggs C. A. (2004). Environmental tolerances of free-living coralline algae (maerl): implications for European marine conservation. *Biological Conservation* 120, 279-289.

Woelkerling W. J. (1985). A taxonomic reassessment of *Spongites* (Corallinaceae, Rhodophyta) based on studies of Kützing's original collections. *British Phycological Journal* 20, 123-153.

Woelkerling W. J. (1988). *The Coralline Red Algae: An Analysis of the Genera and Subfamilies of Nongeniculate Corallinaceae*. Oxford University Press, London, UK.

Woelkerling W. J. & Campbell S. J. (1992). An account of southern Australian species of *Lithophyllum* (Corallinaceae, Rhodophyta). *Bulletin of the British Museum (Natural History), Botany Series* 22, 1-107.

Woelkerling W. J. & Irvine L. M. (1986). The typification and status of *Phymatolithon* (Corallinaceae, Rhodophyta). *British Phycological Journal* 21, 55-80.

Woelkerling W. J. & Lamy D. (1998). *Non-geniculate Coralline Red Algae and the Paris Muséum: Systematics and Scientific History*. Publications Scientifiques du Muséum, Paris, France.

Wolf M. A., Sfriso A., Andreoli C. & Moro I. (2011). The presence of exotic *Hypnea flexicaulis* (Rhodophyta) in the Mediterranean Sea as indicated by morphology, *rbc*L and *cox*1 analyses. *Aquatic Botany* 95, 55-58.

Xie C.-T., Chen C.-S., Ji D.-H. & Xu Y. (2009). Characterization, development and exploitation of EST-derived microsatellites in *Porphyra haitanensis* Chang et Zheng (Bangiales, Rhodophyta). *Journal of Applied Phycology* 21, 367-374.

Xie W., Lewis P. O., Fan Y., Kuo L. & Chen M.-H. (2011). Improving marginal likelihood estimation for

Bayesian phylogenetic model selection. *Systematic Biology* 60, 150-160.

Yakimowski S. B. & Barrett S. C. H. (2014). Clonal genetic structure and diversity in populations of an aquatic plant with combined *vs.* separate sexes. *Molecular Ecology* 23, 2914-2928.

Yang E. C. & Boo S. M. (2006). A red alga-specific phycoerythrin gene for biodiversity surveys of callithamnioid red algae. *Molecular Ecology Notes* 6, 533-535.

Yang E. C., Kim M. S., Geraldino P. J. L., Sahoo D., Shin J.-A. & Boo S. M. (2008). Mitochondrial *cox*1 and plastid *rbc*L genes of *Gracilaria vermiculophylla* (Gracilariaceae, Rhodophyta). *Journal of Applied Phycology* 20, 161-168.

Yang M. Y., Geraldino P. J. L. & Kim M. S. (2013a). DNA barcode assessment of *Gracilaria salicornia* (Gracilariaceae, Rhodophyta) from Southeast Asia. *Botanical Studies* 54, 27 (p. 9).

Yang M. Y., Han E. G. & Kim M. S. (2013b). Molecular identification of *Grateloupia elliptica* and *G. lanceolata* (Rhodophyta) inferred from plastid *rbc*L and mitochondrial COI genes sequence data. *Genes & Genomics* 35, 239-246.

Yang M. Y. & Kim M. S. (2015). Taxonomy of *Grateloupia* (Halymeniales, Rhodophyta) by DNA barcode marker analysis and a description of *Pachymeniopsis volvita* sp. nov. *Journal of Applied Phycology* 27, 1373-1384.

Yang Z. & Rannala B. (2014). Unguided species delimitation using DNA sequence data from multiple loci. *Molecular Biology & Evolution* 31, 3125-3135.

Yoon H. S., Hackett J. D. & Bhattacharya D. (2002). A single origin of the peridinin- and fucoxanthin-containing plastids in dinoflagellates through tertiary endosymbiosis. *Proceedings of the National Academy of Sciences of the United States of America* 99, 11724-11729.

Yoshikawa N., Matsui M. & Inoue-Murayama M. (2013). Characterization of nineteen microsatellite markers for the Japanese clouded salamander, *Hynobius nebulosus*, using the NGS. *Conservation Genetics Resources* 5, 603-605.

Zane L., Bargelloni L. & Patarnello T. (2002). Strategies for microsatellite isolation: a review. *Molecular Ecology* 11, 1-16.

Zhai Z., Zhao W., He C., Yang K., Tang L., Liu S., Zhang Y., Huang Q. & Meng H. (2015). SNP discovery and genotyping using restriction-site-associated DNA sequencing in chickens. *Animal Genetics* 46, 216-219.

Zhakova L. V. (1985). Algae Corallinaceae crustaceae epilithicae in Mari Ochotensi inventae. *Novitates Systematicae Plantarum Non Vascularum* 22, 46-54.

Zhang J., Kapli P., Pavlidis P. & Stamatakis A. (2013). A general species delimitation method with applications to phylogenetic placements. *Bioinformatics* 29, 2869-2876.

Zhang J., Li W., Qu J., Wang X., Liu C. & Liu T. (2015). Development and characterization of microsatellite markers from an enriched genomic library of *Saccharina japonica*. *Journal of Applied Phycology* 27, 479-487.

Zuo Z., Wang C., Cao X., Su Y., Liao L. & Chen Y. (2007). Isolation and characterization of microsatellite loci from a commercial cultivar of *Porphyra haitanensis*. *Molecular Ecology Notes* 7, 522-524.

ANNEX I

Abstract and Poster

This Annex includes the abstract and poster presented in the 6th European Phycological Congress (London, 23-28 August 2015):

Pardo C., Peña V., Bárbara I., Valero M. & Barreiro R. (2015). Poster: A pioneer microsatellite study of clonality in a major maerl-forming species Phymatolithon calcareum (Rhodophyta) in Atlantic Europe. European Journal of Phycology 50 (S1), 128-128.

ITS2 sequences of *Eustigmatos, Chloridella* and *Vischeria* in the Eustigmataceae were well alignable supporting the assumption that they may not be separated genera. Another member of the same group, *Characiopsis saccata*, was not alignable to the former genera. *Characiopsis* was polyphyletic in the ITS2 sequence comparisons. The Monodopsidaceae fell apart in at least two groups, one representing *Monodopsis* and *Pseudotetraedriella*, the other comprising of two separated clades of *Nannochloropsis*. Species of *Monodopsis* and *Vischeria* as currently defined were also distinct in the ITS2 analyses.

1PO.17

A PIONEER MICROSATELLITE STUDY OF CLONALITY IN A MAJOR MAERL-FORMING SPECIES *PHYMATOLITHON CALCAREUM* (RHODOPHYTA) IN ATLANTIC EUROPE

Cristina C Pardo[1] (cristina.pardo.carabias@udc.es), Viviana Peña[1] (vpena@udc.es), Ignacio I Bárbara[1] (barbara@udc.es), Myriam Valero[2] (myriam.valero@sb-roscoff.fr) and Rodolfo R Barreiro[1] (rodbar@udc.es)

[1]BioCost Research Group, Animal Biology, Plant Biology and Ecology Department, A Coruña University, A Coruña 15071, Spain and [2]2CNRS, Sorbonne Universités, UPMC University of Paris 06, UMI EBEA 3614, Evolutionary Biology and Ecology of Algae, PUCCh, UACH Station Biologique de Roscoff, Station Biologique de Roscoff, Roscoff 29688, France

Maerl beds are coastal biogenic habitats formed by accumulations of slow-growing, unattached non-geniculate calcareous algae that harbour a high biodiversity of species in Atlantic Europe. Despite recent conservation efforts to preserve this community at both European and regional scale, several studies have reported serious declines in their extent and quality due to anthropogenic pressures. Appropriate conservation and management of maerl beds requires knowledge of their genetic diversity and connectivity to develop efficient conservation plans. In this context, we show the results of first microsatellite study on the genetic diversity and structure of a major maerl-forming species in Atlantic Europe: *Phymatolithon calcareum* (Pallas) WH Adey & DL McKibbin. Previously, we developed eight species-specific microsatellite markers for this red alga. The study includes samples of fourteen maerl beds along Atlantic Europe from the British Isles to south Portugal. The sampling plan included a detailed study at regional-scale in the rías (i.e. estuaries) of Galicia (NW Spain). Our results show a high, although variable, incidence of clonality in *P. calcareum* even between populations of the same region. Genetic diversity was low but populations were highly differentiated even at regional level. In Galicia, beds from the outer section of the rías are more diverse (lower clonality) than those from intermediate, more sheltered sections. Interestingly, genets were generally not shared between rías while the same genets were shared between populations of the same Ría systematically. All these data will serve to know how *P. calcareum* will evolve and respond to possible disturbances or environmental changes, and therefore will also serve to preserve suitably the maerl beds threatened. Financial support: Ministerio de Ciencia e Innovacion of Spain (CTM2010-18787), Xunta de Galicia (10MMA103003PR) and partially by ASSEMBLE grant agreement no.227799. Cristina Pardo acknowledges financial support by Spain's Ministerio de Educacion (Programa FPU, 2010).

1PO.18

DNA BARCODING OF CONJUGATING GREEN ALGAE - IN SEARCH OF A TOOL TO MAP THE DIVERSITY OF THE ZYGNEMATOPHYCEAE

Florian Mundt (florian.mundt@uni-hamburg.de), Klaus von Schwartzenberg (klaus.von.schwartzenberg@uni-hamburg.de), Dieter Hanelt (dieter.hanelt@uni-hamburg.de) and Hanny Tantau (hanny.tantau@uni-hamburg.de)

Biology, University of Hamburg, Hamburg 22609, Germany

The Zygnematophyceae (= Conjugatophyceae) form a complex group of usually unicellular or filamentous freshwater algae, including more than 3500 species, inhabiting swamps, fenlands and acidic areas. Being sensitive towards changes in those ecosystems Zygnematophycean algae can be used as biological indicators. In the past decades it has been proven that the Zygnematophyceae and land plants share the closest ancestors compared to any other streptophyte algae group. However, scientists often fail to exactly determine Zygnematophyceae on the species level due to the elasticity of phenotypic traits. The influence of DNA barcoding is increasing as a tool for taxonomic determination independent from morphological characteristics. The setup of DNA Barcoding requires reliable genetic material for the generation of proper barcode sequences to be correlated to taxonomically certified strains represented in alga collections. The Microalgae and Zygnematophyceae Collection Hamburg (MZCH) consists of more than 550 taxonomical classified cultures, covering collection localities and species from all over the world. This resource is globally unique and offers the opportunity to test barcode regions recommended by the

A pioneer microsatellite study of clonality in a major maerl-forming species *Phymatolithon calcareum* (Rhodophyta) in Atlantic Europe

Cristina Pardo[1] (cristina.pardo.carabias@udc.es), **Viviana Peña**[1] (vpena@udc.es), **Ignacio Bárbara**[1] (barbara@udc.es), **Myriam Valero**[2] (valero@sb-roscoff.fr) and **Rodolfo Barreiro**[1] (rodbar@udc.es)
[1] Grupo Biocost, Facultade de Ciencias, Universidade da Coruña (Spain). [2] CNRS, Sorbonne Universités, UPMC University of Paris 06, UMI EBEA 3614, Evolutionary Biology and Ecology of Algae, PUCCh, UACH Station Biologique de Roscoff (France).

Introduction

Maerl beds (**Fig. 1**) are coastal biogenic habitats formed by accumulations of slow-growing, unattached non-geniculate calcareous algae that harbor a high biodiversity of species in Atlantic Europe. Despite recent conservation efforts to preserve this community at both European and regional scale, several studies have reported serious declines in their extent and quality due to anthropogenic pressures. As part of two projects financed by Spain and Galicia's Governments (see acknowledgments), specific microsatellite markers[1] have been recently developed for *Phymatolithon calcareum* (Pallas) W. H. Adey & D. L. McKibbin, one of the major maerl-forming species in Atlantic Europe[2,3], affording a suitable tool to develop efficient conservation plans for this species.

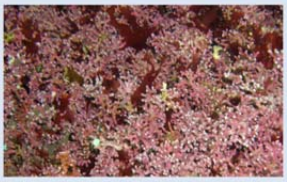


Fig.1. Maerl bed from Galicia (Atlantic Spain).

Objective

To investigate the importance of clonal reproduction and to estimate genetic diversity and connectivity of *P. calcareum* in the maerl beds from Atlantic Europe using population genetics tools.

Material and Methods

Fourteen maerl beds were sampled from the British Isles to Southern Portugal (i.e. more than 1100 individuals collected, **Fig. 2**,). Samples were identified by DNA barcoding and the 455 plants of *P. calcareum* found were genotyped using eight microsatellites[1]. Three sampled beds were located in protected areas: Zara-Shoal (Strangford Lough Marine Conservation Zone), and Illa de Ons and Illas Cíes (Parque Nacional Marítimo-Terreste das Illas Atlánticas de Galicia). In Galicia (**Fig. 2**), a detailed study was carried out in several parts of the "Rías" (i.e. estuaries) according to exposure (external vs. internal parts).
To estimate the importance of clonal reproduction (i.e. fragmentation[4]) we follow the multi-criteria approach based on Multi-Locus Genotypes (MLGs) analyses proposed by Arnaud-Haoand *et al*[5]. In addition, the standard measures of genetic diversity and connectivity were performed.

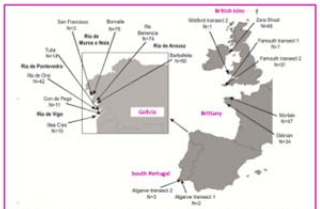


Fig.2. Location of the 14 maerl beds where *P. calcareum* was studied.

Results

Phymatolithon calcareum populations showed a typical signature of clonality since the number of distinct MLGs (***G***) was always smaller than the number of collected samples (***N***), and therefore the clonal diversity (***R***) was smaller than one (**Table 1**). All populations showed private MLGs while shared MLGs were almost only observed among populations located in the same Ría (**Fig. 3**). Even in most populations, evenness among MLGs was not observed (some MLGs are more dominant than others, **Fig. 3**), and the indexes of Simpson (***D****) were greater than 0.7 because of the occurrence of numerous single MLGs in most populations except four (Falmouth, Barbafeita, Benencia and Tulla, **Table 1**). Spatial structure revealed that genotypes were generally intermingled (***Ac*** < 0.08), except in four populations where spatial segregation of clones was more important (0.206 < ***Ac*** < 0.535, **Table 1**).
Low levels of allele (***Na*** / ***AR***) and genetic diversity (***He***, **Table 2**), significant Hardy-Weinberg (H-W) disequilibrium in the form of excess of heterozygotes (F_{IS} < 0, P < 0.05), and significant Linkage Disequilibrium (LD) in almost all populations ($\overline{r_d}$, **Table 2**), showed again a typical signature of clonal reproduction in the *P. calcareum* populations. Genetic differentiation was highly significant among populations (0.040 < ***Fst*** < 0.563, except in three pairs of populations) following a pattern of Isolation By Distance (IBD) at European scale (P = 0.0205). However, a different pattern of genetic differentiation could be revealed by the detailed analysis of connectivity among and within Rías at regional scale: AMOVA showed that variation among Rías of Galicia (≈ 16 %) is significantly higher than among populations within Rías (≈ 7 %).

Table 1. Multi-Locus Genotypes (MLGs) analyses of clonal reproduction in *P. calcareum* populations in maerl beds from Atlantic European coast.

Region	Population	*N*	*G*	*R*	*D**	A_c
British Isles	Zara Shoal	48	33	0.681	0.968	-0.011
	Milford transect 2	1	1	-	-	-
	Falmouth transect 1	1	1	-	-	-
	Falmouth transect 2	31	2	0.033	0.065	-0.500
Brittany	Morlaix	47	17	0.348	0.882	0.083
	Glénan	34	10	0.273	0.774	-0.065
Galicia	San Francisco (ext.)	3	3	-	-	-
	Bornalle (int.)	75	19	0.243	0.708	-0.036
	Barbafeita (ext.)	50	6	0.102	0.580	0.206
	Benencia (int.)	74	4	0.041	0.154	0.212
	Illa de Ons (ext.)	42	27	0.634	0.940	0.062
	Tulla (int.)	14	4	0.231	0.495	0.278
	Illas Cíes (ext.)	15	6	0.357	0.848	0.056
	Con de Pego (int.)	11	5	0.400	0.782	0.535
S. Portugal	Algarve transect 1	2	2	-	-	-
	Algarve transect 2	3	3	-	-	-

N: number of samples. ***G***: clonal genotypic richness, i.e. number of Multi-Locus Genotypes (MLGs). ***R***: genotypic diversity index, *R*= (*G*-1)/(*N*-1), note: *R*=0 all individual share the same genotypes, *R*=1 all individuals have distinct genotypes. ***D****: adapted Simpson's index for clonal genotypic diversity to estimate the clonal heterogeneity, note: *D**=1 the sample is composed of different unique MLGs, *D**=0 the sample is composed of a single repeated MLG. ***Ac***: spatial aggregation index for clonal aggregation, note: *Ac*=1 all neighbours preferentially share the same MLG, *Ac* =0 the probability between nearest neighbours does not differ on average from the global one. In Galicia populations: ext.= external and int.= internal.

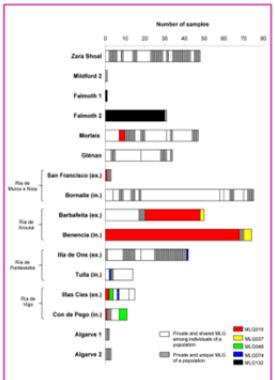


Fig.3. Frequency of MLGs found in *P. calcareum* populations in maerl beds from Atlantic European coast. Private MLGs were indicated in white and grey colour. MLGs that are shared among populations are indicated by the same colour (red, yellow, green, blue and black).

Table 2. Genetic diversity estimates, inbreeding coefficient and linkage disequilibrium for *P. calcareum* populations in maerl beds from Atlantic European coast. Only populations with more than N >10 individuals are shown.

Region	Population	N	Na ± SD (AR ± SD)	Ho ± SD	He ± SD	F_{IS}	$\overline{r_d}$
British Isles	Zara Shoal	48	2.75 ± 1.83 (2.23 ± 0.81)	0.414 ± 0.293	0.454 ± 0.186	0.089	0.070*
Brittany	Morlaix	47	2.13 ± 0.35 (2.07 ± 0.37)	0.367 ± 0.347	0.357 ± 0.165	-0.028	0.091*
	Glénan	34	3.13 ± 2.03 (2.52 ± 1.13)	0.493 ± 0.483	0.358 ± 0.257	-0.384*	0.610*
Galicia	Bornalle (int.)	75	2.88 ± 0.64 (2.52 ± 0.57)	0.418 ± 0.368	0.426 ± 0.163	0.018	0.397*
	Barbafeita (ext)	50	2.25 ± 1.04 (2.05 ± 0.80)	0.420 ± 0.406	0.353 ± 0.247	-0.193*	0.940*
	Benencia (int.)	74	1.63 ± 0.74 (1.41 ± 0.53)	0.370 ± 0.507	0.191 ± 0.260	-0.948*	-0.030
	Illa de Ons (ext.)	42	3.13 ± 0.83 (3.02 ± 0.74)	0.458 ± 0.314	0.558 ± 0.147	0.180	0.146*
	Tulla (int.)	14	2.75 ± 0.71 (2.61 ± 0.63)	0.705 ± 0.426	0.455 ± 0.220	-0.584*	0.386*
	Illas Cíes (ext.)	15	3.50 ± 1.07 (3.46 ± 1.04)	0.558 ± 0.387	0.581 ± 0.161	0.040	0.447*
	Con de Pego (int.)	11	1.88 ± 0.64 (1.88 ± 0.64)	0.466 ± 0.501	0.343 ± 0.247	-0.385*	0.490*

N: number of samples. ***No***: number of alleles; brackets denote alleleic richness (***AR***) recalculated using a rarefaction method with 11 samples. ***SD***: standard deviation. ***Ho***: observed heterozygosity. ***He***: unbiased expected heterozygosity. F_{IS}: inbreeding coefficient. $\overline{r_d}$: coefficient of Multi-Locus Linkage Disequilibrium. *Significant values (P < 0.05). In Galicia populations: ext.= external and int.= internal.

Conclusions

- Clonality is important in *Phymatolithon calcareum* but highly variable among populations even within the same region. The differences in clonality are thus not related with the geographic or latitudinal distribution of populations.
- There are two populations in the same Ría (Ría de Arousa: Barbafeita and Benencia) that exhibited low diversity (*R*) and heterogeneity (*D**), and further studies are needed to explain what are the main drivers of the clonality in this site. Studies about this question are also necessary in Falmouth, where *R* and *D** were the lowest.
- Other two populations located in the British Isles and Galicia (Zara Shoal and Illa de Ons, respectively) showed the highest diversity (*R*) and heterogeneity (*D**). Both maerl beds are located in protected areas but also under high hydrodynamic conditions (wave exposure, currents). Studies about this question are necessary.
- At regional scale, the comparison of populations between and within Rías of Galicia reveals that MLGs are generally not shared between Rías, suggesting that dispersal is restricted. However, the same MLGs were systematically shared within the same Ría, suggesting that fragmentation could be the main propagation mechanism at local level. Besides, external parts of the Rías are more diverse (lower clonality) than those from internal, sheltered sections.
- The results of this study demonstrate that an assessment of the populations at genetic level is necessary before performing any conservation action that involve transplantation of plants among beds even at local level.

Acknowledgements
Financial support: Ministerio de Ciencia e Innovación of Spain (grant CTM2010-18787, partially co-founded by FEDER, Fondo Europeo de Desarrollo Regional), and Xunta de Galicia (grant 10MMA103003PR). 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).
Cristina Pardo acknowledges fellowship from Spain's Ministerio de Educación (Programa FPU, 2010) and their program grant to do stays (Programa FPU Estancias breves y traslados temporales, 2013). Viviana Peña acknowledges fellowship from Xunta de Galicia (Axudas de apoio á etapa inicial de formación posdoutoral, 2012).

References
1. Pardo *et al.* (2014). *Phycologia* 53, 474-479. 2. Pardo *et al.* (2014). *PLoS ONE* 9, E104073. doi: 10.1371/journal.pone.0104073. 3. Carro *et al.* (2014). *Phytotaxa* 190: 176-189. 4. Peña *et al.* (2014). *Cryptogamie, Algologie* 35: 15-25. 5. Arnaud-Haond *et al.* (2007). *Molecular Ecology* 16, 5115-5139.

ANNEX II

Extended Summary (Spanish)

DESARROLLO Y APLICACIÓN DE MARCADORES MOLECULAR A ESPECIES FORMADORAS DE MAERL EN EUROPA: DATOS PARA SU CONSERVACIÓN

INTRODUCCIÓN

1. Biología de la Conservación: protegiendo las especies

La Biología de la Conservación aborda el estudio de la biología de las especies, las comunidades y los ecosistemas sometidos a perturbación, y su objetivo es proporcionar principios y herramientas para el mantenimiento de la biodiversidad (Soulé 1985). Para ello se requiere un conocimiento adecuado de las poblaciones de las distintas especies que conviven en el tiempo y en el espacio. Sin embargo, el primer problema surge en la propia definición de especie, debido a que hasta ahora no se ha logrado un consenso sobre qué criterio debe seguir dicha definición (de Queiroz 2007). En el caso particular de las algas, las especies se han delimitado convencionalmente mediante morfología y/o compatibilidad sexual (Leliaert *et al.* 2014). Sin embargo, esta aproximación está cambiando, particularmente en el caso de Rhodophyta, un grupo que presenta una alta plasticidad y una distribución ecológica compartida, y donde el ciclo de vida de muchas de sus especies sólo se conoce parcialmente (Irvine & Chamberlain 1994). Actualmente, para delimitar las distintas especies de algas rojas se está utilizando información molecular.

Uno de los métodos más populares que utiliza información molecular para delimitar especies es el *DNA barcoding* (http://www.ibol.org; Ratnasingham & Hebert 2007). Esta técnica se utiliza también para detectar especies crípticas (por ejemplo, Kucera & Saunders 2012, Milstein & Saunders 2012), y para ayudar en la detección de nuevas especies (por ejemplo, Hind & Saunders 2013a,b). En algas rojas, el *DNA barcoding* ya ha sido ampliamente utilizado (por ejemplo, Saunders 2005, Saunders & McDevit 2012b). Para aplicar este método a grandes conjuntos de datos con el objeto de delimitar especies, existen varias herramientas bioinformáticas basadas en distancias genéticas. Entre ellas destaca ABGD ("Automatic Barcode Gap Discovery", Puillandre *et al.* 2012a). Esta herramienta se ha utilizado en el capítulo 1 de la presente tesis para evaluar la diversidad de especies de algas rojas coralinas formadoras de maerl en el Atlántico Europeo, proporcionando con ello uno de los pocos ejemplos en algas marinas donde los *DNA barcodes* se han usado para delimitar especies con ABGD (Pardo *et al.* 2014a).

Otro método para detectar especies basándose en información molecular es GMYC ("General Mixed Yule Coalescent model"), que ha sido ampliamente utilizado en muchos grupos taxonómicos, pero raramente en algas marinas (Payo *et al.* 2013, Vieira *et al.* 2014, Peña *et al.* 2015a). En el capítulo 1 de esta tesis, GMYC sirvió como segundo criterio para delimitar especies formadoras de maerl (Pardo *et al.* 2014a), siguiendo la recomendación actual de usar simultáneamente más de un criterio para mejorar la confianza en la delimitación de especies. Por otra parte, en el capítulo 2, GMYC también se utilizó para describir una nueva especie mayoritaria del maerl en el Atlántico Ibérico (Peña *et al.* 2015b).

Una vez que las especies de una zona son delimitadas, el siguiente paso es preservar su diversidad, lo que implica proteger la diversidad genética de sus poblaciones, ya que es la materia prima para que las poblaciones evolucionen en respuesta a los cambios ambientales (Frankham 1996), y su pérdida aumenta el riesgo de extinción (Reed & Frankham 2003, Allendorf *et al.* 2013). La diversidad genética se puede estimar con diversos marcadores cuantitativos (por ejemplo, QTLs) y moleculares (Frankham *et al.* 2002, Allendorf *et al.* 2013). Entre los últimos destacan los microsatélites, cuya utilidad para estudiar la diversidad genética y flujo de genes (es decir, la conectividad) es bien conocida (Selkoe & Toonen 2006, Arif *et al.* 2011, Krueger-Hadfield *et al.* 2011, Oppliger *et al.* 2014). En el caso concreto de las algas marinas, el desarrollo de microsatélites ha recibido poca atención, especialmente en Rhodophyta (Andreakis *et al.* 2007). Probablemente, la razón es que estos marcadores suelen ser especie-específicos, por lo que deben ser aislados *de novo* para cada especie (Zane *et al.* 2002). La tecnología de secuenciación de nueva generación (NGS, "Next Generation Sequencing") ha facilitado el aislamiento de microsatélites a gran escala en especies no modelo (Schoebel *et al.* 2013). En la presente tesis se aplicó por primera vez esta tecnología a un alga coralina para desarrollar microsatélites que permitan evaluar la diversidad genética y la estructura de población, tomando como objeto de estudio una especie formadora de maerl (*Phymatolithon calcareum;* ver capítulo 4 y Anexo I, Pardo *et al.* 2014b, 2015b).

2. Los fondos de maerl en el Atlántico Europeo

Los fondos de maerl son acúmulos de algas rojas coralinas no geniculadas (es decir, carentes de uniones flexibles no calcificadas) que crecen libres sobre el fondo marino de zonas costeras, en forma de pequeñas masas nodulares más o menos ramificadas. En estos acúmulos, las ramas de los nódulos se entrecruzan formando una matriz tridimensional biogénica y frágil, que sirve de hábitat para muchas algas, invertebrados y larvas. Además, los

fondos de maerl también actúan como áreas de reclutamiento para especies de valor comercial (BIOMAERL Team 2003, Kamenos *et al.* 2004a-d). Todo ello hace que los fondos de maerl contengan una biodiversidad elevada e inusual (algunas especies son endémicas de este tipo de hábitat), por lo que se consideran *hotspots* de diversidad marina (Foster 2001, Nelson 2009, Hall-Spencer *et al.* 2010, Peña *et al.* 2014a). En el Atlántico Europeo, los bancos de maerl están presentes desde Svalbard hasta el sur de Portugal, así como en la región de Macaronesia, encontrándose en una amplia variedad de condiciones hidrodinámicas y batimétricas, desde el intermareal hasta los 60 m de profundidad (Grall 2003, Hall-Spencer *et al.* 2010, Peña Freire 2010, Peña *et al.* 2014a).

A pesar de su gran valor ecológico, el maerl ha sido explotado con varios fines, particularmente en la Bretaña francesa y en las Islas Británicas (Briand 1991, Birkett *et al.* 1998, Grall 2003, Grall & Hall-Spencer 2003). Su principal destino ha sido el de servir como enmienda para corregir la acidez del suelo. A menor escala, también se ha utilizado con otros fines industriales. Esta explotación directa, junto con el impacto negativo indirecto de otras actividades antrópicas (por ejemplo, la pesca de arrastre, los residuos generados por la acuicultura, las cadenas de amarre, la eutrofización, la presencia de especies invasoras, etc.) ha provocado una degradación de la estructura y diversidad de los fondos de maerl del Atlántico Europeo (BIOMAERL Team 2003, Peña & Bárbara 2008a, Hall-Spencer *et al.* 2010, Peña Freire 2010). De hecho, varios estudios detectaron que los fondos de maerl han disminuido, tanto en extensión como en calidad, a lo largo de toda la costa europea (BIOMAERL Team 2003, Peña & Bárbara 2008a, Hall-Spencer *et al.* 2010). Por otra parte, las predicciones realizadas para futuros escenarios de calentamiento global y acidificación de los océanos, han puesto de manifiesto que los fondos de maerl del Atlántico Noreste probablemente sufrirán un nuevo declive, fundamentalmente debido a que su naturaleza calcárea los hace especialmente vulnerables a descensos del pH del agua de mar (Nelson 2009, Büdenbender *et al.* 2011, Noisette *et al.* 2013a,b, Brodie *et al.* 2014, McCoy & Kamenos 2015). Resulta sorprendente que un hábitat biogénico tan relevante y sometido a agresiones intensas, apenas haya recibido protección legal en Europa. Sobre todo, si tenemos presente que la reducida velocidad de crecimiento del maerl hace que sus poblaciones se consideren un recurso no renovable que requiere la consiguiente protección para su conservación. La única cobertura legal existente se centra en dos especies consideradas entre las principales formadoras de maerl en Europa, *Phymatolithon calcareu*m y *Lithothamnion corallioides*, que están parcialmente protegidas por estar incluidas en el Anexo V de la Directiva Hábitats (DH) de la Unión Europea (UE). Este anexo

enumera especies cuya explotación debe ser compatible con el mantenimiento de un estado de conservación favorable, aunque en ningún momento prohibe su explotación. Por otra parte, los fondos de maerl no están explícitamente recogidos con ese nombre entre los hábitats de interés comunitario del Anexo I de la DH. Sin embargo, algunos estados miembros de la UE los consideran implícitamente incluídos en las categorías del Anexo I "bancos de arena ligeramente cubiertos por el mar durante todo el tiempo" y "grandes estuarios y bahías someras". Además, la Comisión OSPAR acordó en 2004 incluir a los bancos de maerl en su Lista de Especies y Hábitats Amenazados y/o en Declive a la vista de las evidencias de su deterioro y presiones a las que están sometidos. Por otro lado, los fondos de maerl han sido incluidos en la Red Natura 2000 con el objetivo de conservar su alto valor biológico, y están catalogados en el Sistema Europeo de Información sobre la Naturaleza (EUNIS).

En cuanto a las propias especies formadoras de maerl, convencionalmente su identificación se ha llevado a cabo mediante estudios taxonómicos basados en rasgos morfológicos. Por ello, anteriormente a esta tesis no existe ninguna evaluación de la diversidad de especies coralinas formadoras de maerl en el Atlántico Europeo desde un punto de vista molecular, a pesar de que varios estudios ya indicaban que las algas coralinas podían ocultar una considerable diversidad críptica, debido a su alta plasticidad fenotípica y a la falta de características diagnóstico (por ejemplo, Robba *et al.* 2006, Walker *et al.* 2009). Con la sospecha de que bajo las especies formadoras de maerl también podía estar oculta una diversidad críptica, el punto de partida de esta tesis fue realizar una evaluación molecular de su diversidad específica (capítulo 1 y capítulo 2), ya que no podemos evaluar adecuadamente en qué medida los fondos de maerl encontrados en un área del Atlántico Europeo son equivalentes a las que se encuentran en otras áreas, sin un conocimiento exacto del número real de las especies formadoras que se encuentran en cada región.

En cuanto al ciclo de vida de las especies formadoras de maerl, la información es escasa y, en general, se considera que su principal modo de reproducción es asexual mediante fragmentación (Bosence 1976, Johansen 1981). Los escasos registros de estructuras reproductoras (conceptáculos) en dichas especies sostienen esta hipótesis (Suneson 1958, Adey & McKibbin 1970, Woelkerling & Irvine 1986, Irvine & Chamberlain 1994, Peña & Bárbara 2004, 2008b). Además, en el Atlántico Europeo, la fase gametofítica es más difícil de encontrar, con una única observación en dos especies formadoras de maerl (*Phymatolithon calcareum* y *Lithothamnion corallioides*), ambas bajo formas costrosas e identificadas exclusivamente por medio de morfología (Cabioch 1969, 1970, Mendoza & Cabioch 1998). Las

herramientas moleculares disponibles en la actualidad proporcionan una nueva perspectiva para realizar estudios más fiables sobre la reproducción de las coralinas formadoras de maerl, especialmente de su fase gametofítica. Por ello, en esta tesis se ha estudiado con detalle esta fase en una de las principales especies formadoras de maerl (*P. calcareum*) desde un enfoque molecular (capítulo 3).

OBJETIVOS

Para llevar a cabo una adecuada gestión de los fondos de maerl se necesita información sobre múltiples aspectos: distribución, diversidad de las especies asociadas, estado de conservación, etc. Pero también son necesarios estudios detallados sobre la diversidad específica de sus especies formadoras, su ciclo de vida y la diversidad genética de sus poblaciones. En el momento en el que comenzaron los estudios de esta tesis doctoral, y como se ha visto a lo largo de la introducción, sólo los dos primeros aspectos habían sido abordados, pero siempre bajo una perspectiva morfológica (por ejemplo, Adey & McKibbin 1970, Cabioch 1970, Afonso-Carrillo & Gil-Rodríguez 1982, Irvine & Chamberlain 1994, Mendoza & Cabioch 1998). Esta tesis ha dado un paso adelante en el conocimiento de los fondos de maerl del Atlántico Europeo, usando por primera vez marcadores moleculares para resolver las cuestiones citadas anteriormente. De esta manera, los objetivos planteados fueron:

- Valorar la diversidad real de las especies formadoras de maerl en los fondos del Atlántico Europeo.
- Investigar las características reproductivas sexuales en una de las principales especies formadoras de maerl de la costa Atlántica Europea: *Phymatolithon calcareum*.
- Desarrollar marcadores moleculares especie-específicos con poder de resolución apropiado para estudios poblacionales (es decir, microsatélites) en *P. calcareum* utilizando la tecnología de secuenciación de nueva generación (NGS), con el objeto final de aplicarlos a varias poblaciones de esta especie en fondos de maerl de la costa Atlántica Europea.

RESULTADOS Y CONCLUSIONES

En el capítulo 1 de esta tesis (Pardo *et al.* 2014a), las especies formadoras de maerl del Atlántico Europeo, particularmente del área OSPAR, fueron delimitadas utilizando un enfoque taxonómico integrador (Schlick-Steiner *et al.* 2010). De esta forma, se combinó información morfológica y biogeográfica con datos de secuencias de los genomas mitocondrial (la región del gen COI-5P generalmente utilizada como *DNA barcode* en estudios de algas rojas y de otros

grupos taxonómicos) y plastidial (*psb*A, un marcador usado con frecuencia en estudios filogenéticos dentro de la clase Florideophyceae). Además, la información molecular utilizada incluyó secuencias obtenidas de materiales tipo de especies formadoras de maerl, como el neotipo de *Phymatolithon calcareum* y el holotipo de *Mesophyllum sphaericum*. Para delimitar las especies, se usó la herramienta bioinformática ABGD basada en distancias, y GMYC como método probabilístico basado en el modelo de coalescencia. Paralelamente, se llevó a cabo una revisión de toda la bibliografía concerniente a las especies formadoras de maerl en el Atlántico Europeo, que se remonta hasta principios del siglo XX, centrándose tanto en las descripciones de especies como en su distribución. Como resultado de este estudio, se delimitaron 13 taxones con rangos biológicos plausibles, a partir de un total de 224 especímenes recolectados desde Svalbard hasta las Islas Canarias. De estos taxones, 6 se asignaron a especies ya descritas: *Lithothamion glaciale, L. corallioides, Phymatolithon calcareum*, *Mesophyllum sphaericum*, *Lithophyllum fasciculatum* y *L. dentatum.* Más recientemente, *L. fasciculatum* así como colecciones de Irlanda y Bretaña identificadas como *L. dentatum* han sido consideradas conespecíficos de *L. incrustans* (Hernández-Kantún *et al.* 2015a). Los resultados de este capítulo indican que la mayoría de las especies que forman maerl tienen pequeñas áreas de distribución y se observa un reemplazo gradual de las especies con la latitud. De esta manera, las latitudes frías del Atlántico Europeo están dominadas por dos miembros del género *Lithothamnion—L. glaciale* y otra especie pendiente de asignar nombre (*Lithothamnion* sp.1)—*,* mientras que *L. corallioides* y *P. calcareum* son los principales constituyentes de los fondos de maerl en la Bretaña francesa y en el Canal de la Mancha. En el Atlántico Ibérico, *P. calcareum* y *L. corallioides* coexisten con otra especie (*Phymatolithon* sp. 3) que llega a ser dominante en los fondos de maerl del sur de Portugal (Carro *et al.* 2014, Pardo *et al.* 2014a). Este estudio ha supuesto un avance significativo en el conocimiento del maerl del Atlántico Europeo, ya que es la primera vez que un enfoque molecular se utiliza para llevar a cabo una evaluación de la diversidad específica de sus especies formadoras.

En el capítulo 2 se abordó la descripción de la nueva especie formadora de maerl mayoritaria en el Atlántico Ibérico, encontrada durante el estudio llevado a cabo en el capítulo 1 (i.e. *Phymatolithon* sp. 3), demostrando que también en los fondos de maerl subyace una fuerte diversidad críptica (Peña *et al.* 2015b). Finalmente, esta nueva especie fue descrita como *Phymatolithon lusitanicum*. Además, en el capítulo 2, se realizó un estudio comparativo de esta especie con otros miembros europeos del género *Phymatolithon.* Dicho análisis incluyó

la obtención de información molecular del material tipo de varias especies, como *Phymatolithon lamii* y *P. laevigatum.* Aunque *P. lusitanicum* es especialmente abundante en fondos submareales del Atlántico Ibérico, también se detectó como maerl en el intermareal irlandés y en el Mar Mediterráneo Occidental (Mar de Alborán, Islas Baleares) hasta los 64 m de profundidad. Hasta ahora, *P. lusitanicum* ha sido encontrado únicamente formando maerl. Por el contrario, *P. lamii* (una especie anatómicamente próxima) sólo se ha registrado bajo forma incrustante, incluso en muestras recogidas asociadas a fondos de maerl. Dentro de este estudio, también descubrimos que el análisis molecular del material tipo de la especie incrustante *Lithothamnion hamelii* indica su conespecificidad con *P. calcareum.*

En el capítulo 3 se realizó una contribución al conocimiento del ciclo de vida de una de las principales especies formadoras de maerl: *Phymatolithon calcareum* (Peña *et al.* 2014b). De esta manera, se confirmó con información molecular (usando secuencias del gen COI-5P), la presencia de gametófitos de esta especie bajo formas incrustantes. Cabe mencionar que la identificación de las muestras de *P. calcareum* fue corroborada utilizando una secuencia del neotipo de esta especie, y también con otras secuencias de *P. calcareum* obtenidas de especímenes de maerl procedentes de varias regiones del Atlántico Europeo. Las muestras incrustantes estudiadas procedían de un banco de maerl submareal de la Bretaña francesa, curiosamente de la misma zona en la que Mendoza & Cabioch (1998) citaron por primera vez la presencia de gametófitos de *P. calcareum* y *Lithothamion corallioides,* también bajo formas incrustantes, usando datos morfológicos. Adicionalmente, también se realizó un estudio morfológico de estas colecciones mediante microscopio electrónico de barrido (SEM, "Scanning Electron Microscope"), concluyendo que eran gametófitos femeninos a pesar de encontrar conceptáculos vacíos. La escasez de estructuras reproductoras asexuales en el maerl, junto con la presencia de gametófitos bajo formas costrosas, lleva a sugerir que el modo de propagación dominante es vegetativo por simple fragmentación. Hay que destacar que este estudio es el primero que confirma la presencia de gametófitos de *P. calcareum* bajo formas incrustantes en base a información molecular.

En el último capítulo de esta tesis (capítulo 4) se presenta el primer conjunto de marcadores microsatélite desarrollados para un alga roja coralinácea (Pardo *et al.* 2014b). En concreto, se desarrollaron ocho loci polimórficos (tres dinucleótidos, dos trinucleótidos y tres tetranucleótidos) para *Phymatolithon calcareum*, utilizando la tecnología NGS. En estos loci se detectó un bajo polimorfismo, pero no parece ser una característica exclusiva de este tipo de alga coralina, ya que en otras algas rojas donde se han desarrollado microsatélites, los valores

mostrados también fueron bajos, sugieriendo que el bajo polimorfismo parece una característica típica de las algas rojas en general. Por otra parte, los ocho loci fueron incorporados con éxito en un protocolo de PCR multiplex para facilitar su posterior genotipado. Los microsatélites desarrollados proporcionan una herramienta adecuada para realizar estudios sobre la genética poblacional de las especies formadoras de maerl, con el fin de incorporar dicha información a los planes de conservación y gestión desarrollados hasta ahora en estos hábitats de alto valor para la conservación. A este respecto, en el Anexo I de esta tesis (Pardo *et al.* 2015b), se muestran los primeros resultados obtenidos tras la aplicación de estos ocho microsatélites a varias poblaciones de *P. calcareum* en fondos de maerl del Atlántico Europeo, en donde se detectó una fuerte señal de clonalidad, aunque variable, incluso en poblaciones situadas en la misma región.

ANNEX III

Extended Summary (Galician)

DESENVOLVEMENTO E APLICACIÓN DE MARCADORES MOLECULAR A ESPECIES FORMADORAS DE MAERL EN EUROPA: DATOS PARA A SÚA CONSERVACIÓN

INTRODUCCIÓN

1. Bioloxía da Conservación: protexendo as especies

A Bioloxía da Conservación aborda o estudo da bioloxía das especies, as comunidades e os ecosistemas sometidos a perturbación, e o seu obxectivo é proporcionar principios e ferramentas para o mantemento da biodiversidade (Soulé 1985). Para iso requírese un coñecemento adecuado das poboacións das distintas especies que conviven no tempo e no espazo. Con todo, o primeiro problema xorde na propia definición de especie, debido a que ata agora non se logrou un consenso sobre que criterio debe seguir dita definición (de Queiroz 2007). No caso particular das algas, as especies delimitáronse convencionalmente mediante morfoloxía e/ou compatibilidade sexual (Leliaert *et al.* 2014). Con todo, esta aproximación está a cambiar, particularmente no caso de Rhodophyta, un grupo que presenta unha alta plasticidade e unha distribución ecolóxica compartida, e onde o ciclo de vida de moitas das súas especies só se coñece parcialmente (Irvine & Chamberlain 1994). Actualmente, para delimitar as distintas especies de algas vermellas está a empregarse información molecular.

Un dos métodos máis populares que utiliza información molecular para delimitar especies é o *DNA barcoding* (http://www.ibol.org; Ratnasingham & Hebert 2007). Esta técnica utilízase tamén para detectar especies crípticas (por exemplo, Kucera & Saunders 2012, Milstein & Saunders 2012), e para axudar na detección de novas especies (por exemplo, Hind & Saunders 2013a,b). En algas vermellas, o *DNA barcoding* xa foi amplamente empregado (por exemplo, Saunders 2005, Saunders & McDevit 2012b). Para aplicar este método a grandes conxuntos de datos co obxecto de delimitar especies, existen varias ferramentas bioinformáticas baseadas en distancias xenéticas. Entre elas destaca ABGD ("Automatic Barcode Gap Discovery", Puillandre *et al.* 2012a). Esta ferramenta utilizouse no capítulo 1 da presente tese para avaliar a diversidade de especies de algas vermellas coralinas formadoras de maerl no Atlántico Europeo, proporcionando con iso un dos poucos exemplos en algas mariñas onde os *DNA barcodes* usáronse para delimitar especies con ABGD (Pardo *et al.* 2014a).

Outro método para detectar especies baseándose en información molecular é GMYC ("General Mixed Yule Coalescent model"), que foi amplamente empregado en moitos grupos

taxonómicos, pero raramente en algas mariñas (Payo *et al.* 2013, Vieira *et al*. 2014, Peña *et al.* 2015a). No capítulo 1 desta tese, GMYC serviu como segundo criterio para delimitar especies formadoras de maerl (Pardo *et al.* 2014a), seguindo a recomendación actual de usar simultaneamente máis dun criterio para mellorar a confianza na delimitación de especies. Por outra banda, no capítulo 2, GMYC tamén se utilizou para describir unha nova especie maioritaria do maerl no Atlántico Ibérico (Peña *et al.* 2015b).

Unha vez que as especies dunha zona son delimitadas, o seguinte paso é preservar a súa diversidade, o que implica protexer a diversidade xenética das súas poboacións, xa que é a materia prima para que as poboacións evolucionen en resposta aos cambios ambientais (Frankham 1996), e a súa perda aumenta o risco de extinción (Reed & Frankham 2003, Allendorf *et al.* 2013). A diversidade xenética pódese estimar con diversos marcadores cuantitativos (por exemplo, QTLs) e moleculares (Frankham *et al.* 2002, Allendorf *et al*. 2013). Entre os últimos destacan os microsatélites, nos que a súa utilidade para estudar a diversidade xenética e fluxo de xenes (é dicir, a conectividade) é ben coñecida (Selkoe & Toonen 2006, Arif *et al.* 2011, Krueger-Hadfield *et al.* 2011, Oppliger *et al.* 2014). No caso concreto das algas mariñas, o desenvolvemento de microsatélites recibiu pouca atención, especialmente en Rhodophyta (Andreakis *et al*. 2007). Probablemente, a razón é que estes marcadores adoitan ser especie-específicos, polo que deben ser illados *de novo* para cada especie (Zane *et al.* 2002). A tecnoloxía de secuenciación de nova xeración (NGS, "Next Generation Sequencing") facilitou o illamento de microsatélites a gran escala en especies non modelo (Schoebel *et al.* 2013). Na presente tese aplicouse por primeira vez esta tecnoloxía a un alga coralina para desenvolver microsatélites que permitan avaliar a diversidade xenética e a estrutura de poboación, tomando como obxecto de estudo unha especie formadora de maerl (*Phymatolithon calcareum*; ver capítulo 4 e Anexo I, Pardo *et al.* 2014b, 2015b).

2. Os fondos de maerl no Atlántico Europeo

Os fondos de maerl son acúmulos de algas vermellas coralinas non xeniculadas (é dicir, carentes de unións flexibles non calcificadas) que crecen libres sobre o fondo mariño de zonas costeiras, en forma de pequenas masas nodulares máis ou menos ramificadas. Nestes acúmulos, as ramas dos nódulos entrecrúzanse formando unha matriz tridimensional bioxénica e fráxil, que serve de hábitat para moitas algas, invertebrados e larvas. Ademáis, os fondos de maerl tamén actúan como áreas de recrutamento para especies de valor comercial (BIOMAERL Team 2003, Kamenos *et al.* 2004a-d). Todo iso fai que os fondos de maerl

conteñan unha biodiversidade elevada e inusual (algunhas especies son endémicas deste tipo de hábitat), polo que se consideran *hotspots* de diversidade mariña (Foster 2001, Nelson 2009, Hall-Spencer *et al.* 2010, Peña *et al.* 2014a). No Atlántico Europeo, os bancos de maerl están presentes dende Svalbard ata o sur de Portugal, así como na rexión de Macaronesia, atopándose nunha ampla variedade de condicións hidrodinámicas e batimétricas, dende o intermareal ata os 60 m de profundidade (Grall 2003, Hall-Spencer *et al.* 2010, Peña Freire 2010, Peña *et al.* 2014a).

A pesar do seu gran valor ecolóxico, o maerl foi explotado con varios fins, particularmente na Bretaña francesa e nas Illas Británicas (Briand 1991, Birkett *et al.* 1998, Grall 2003, Grall & Hall-Spencer 2003). O seu principal destino foi o de servir como emenda para corrixir a acidez do chan. A menor escala, tamén se empregou con outros fins industriais. Esta explotación directa, xunto co impacto negativo indirecto doutras actividades antrópicas (por exemplo, a pesca de arrastre, os residuos xerados pola acuicultura, as cadeas de amarre, a eutrofización, a presenza de especies invasoras, etc.) provocou unha degradación da estrutura e diversidade dos fondos de maerl do Atlántico Europeo (BIOMAERL Team 2003, Peña & Bárbara 2008a, Hall-Spencer *et al.* 2010, Peña Freire 2010). De feito, varios estudos detectaron que os fondos de maerl diminuíron, tanto en extensión como en calidade, ao longo de toda a costa europea (BIOMAERL Team 2003, Peña & Bárbara 2008a, Hall-Spencer *et al.* 2010). Por outra banda, as predicións realizadas para futuros escenarios de quecemento global e acidificación dos océanos, puxeron de manifesto que os fondos de maerl do Atlántico Nordés probablemente sufrirán un novo declive, fundamentalmente debido a que a súa natureza calcárea fainos especialmente vulnerables a descensos do pH da auga de mar (Nelson 2009, Büdenbender *et al.* 2011, Noisette *et al.* 2013a,b, Brodie *et al.* 2014, McCoy & Kamenos 2015). Resulta sorprendente que un hábitat bioxénico tan relevante e sometido a agresións intensas, apenas recibise protección legal en Europa. Sobre todo, se temos presente que a reducida velocidade de crecemento do maerl fai que as súas poboacións sexan consideradas un recurso non renovable que require a consecuente protección para a súa conservación. A única cobertura legal existente céntrase en dúas especies consideradas entre as principais formadoras de maerl en Europa, *Phymatolithon calcareum* e *Lithothamnion corallioides*, que están parcialmente protexidas por estar incluídas no Anexo V da Directiva Hábitats (DH) da Unión Europea (UE). Este anexo enumera especies nas que a súa explotación debe ser compatible co mantemento dun estado de conservación favorable, aínda que en ningún momento prohibe a súa explotación. Por outra banda, os fondos de maerl non están

explicitamente recollidos con ese nome entre os hábitats de interese comunitario do Anexo I da DH. Con todo, algúns estados membros da UE considéranos implicitamente incluídos nas categorías do Anexo I "bancos de area lixeiramente cubertos polo mar durante todo o tempo" e "grandes estuarios e baías someras". Ademáis, a Comisión OSPAR acordou en 2004 incluír aos bancos de maerl na súa Lista de Especies e Hábitats Ameazados e/ou en Declive á vista das evidencias da seu deterioro e polas presións ás que están sometidos. Doutra banda, os fondos de maerl foron incluídos na Rede Natura 2000 co obxectivo de conservar o seu alto valor biolóxico, e están catalogados no Sistema Europeo de Información sobre a Natureza (EUNIS).

En canto ás propias especies formadoras de maerl, convencionalmente a súa identificación levouse a cabo mediante estudos taxonómicos baseados en trazos morfolóxicos. Por iso, anteriormente a esta tese non existe ningunha avaliación da diversidade de especies coralinas formadoras de maerl no Atlántico Europeo dende un punto de vista molecular, a pesar de que varios estudos xa indicaban que as algas coralinas podían ocultar unha considerable diversidade críptica, debido á súa alta plasticidade fenotípica e á falta de características diagnóstico (por exemplo, Robba *et al.* 2006, Walker *et al.* 2009). Coa sospeita de que baixo as especies formadoras de maerl tamén podía estar oculta unha diversidade críptica, o punto de partida desta tese foi realizar unha avaliación molecular da súa diversidade específica (capítulo 1 e capítulo 2), xa que non podemos avaliar adecuadamente en que medida os fondos de maerl atopados nunha área do Atlántico Europeo son equivalentes ós que se atopan noutras áreas, sen un coñecemento exacto do número real das especies formadoras que se atopan en cada rexión.

En canto ao ciclo de vida das especies formadoras de maerl, a información é escasa e, en xeral, considérase que o seu principal modo de reprodución é asexual mediante fragmentación (Bosence 1976, Johansen 1981). Os escasos rexistros de estruturas reprodutoras (conceptáculos) nas devanditas especies sosteñen esta hipótese (Suneson 1958, Adey & McKibbin 1970, Woelkerling & Irvine 1986, Irvine & Chamberlain 1994, Peña & Bárbara 2004, 2008b). Ademáis, no Atlántico Europeo, a fase gametofítica é máis difícil de atopar, cunha única observación en dúas especies formadoras de maerl (*Phymatolithon calcareum* e *Lithothamnion corallioides*), ambas baixo formas costrosas e identificadas exclusivamente por medio de morfoloxía (Cabioch 1969, 1970, Mendoza & Cabioch 1998). As ferramentas moleculares dispoñibles na actualidade proporcionan unha nova perspectiva para realizar estudos máis fiables sobre a reprodución das coralinas formadoras de maerl, especialmente da

súa fase gametofítica. Por iso, nesta tese estudouse con detalle esta fase nunha das principais especies formadoras de maerl (*P. calcareum*) dende un enfoque molecular (capítulo 3).

OBXECTIVOS

Para levar a cabo unha adecuada xestión dos fondos de maerl necesítase información sobre múltiples aspectos: distribución, diversidade das especies asociadas, estado de conservación, etc. Pero tamén son necesarios estudos detallados sobre a diversidade específica das súas especies formadoras, o seu ciclo de vida e a diversidade xenética das súas poboacións. No momento no que comezaron os estudos desta tese doutoral, e como se viu ao longo da introdución, só os dous primeiros aspectos foran abordados, pero sempre baixo unha perspectiva morfolóxica (por exemplo, Adey & McKibbin 1970, Cabioch 1970, Afonso-Carrillo & Gil-Rodríguez 1982, Irvine & Chamberlain 1994, Mendoza & Cabioch 1998). Esta tese deu un paso adiante no coñecemento dos fondos de maerl do Atlántico Europeo, usando por primeira vez marcadores moleculares para resolver as cuestións citadas anteriormente. Desta maneira, os obxectivos expostos foron:

- Valorar a diversidade real das especies formadoras de maerl nos fondos do Atlántico Europeo.
- Investigar as características reprodutivas sexuais nunha das principais especies formadoras de maerl da costa Atlántica Europea: *Phymatolithon calcareum*.
- Desenvolver marcadores moleculares especie-específicos con poder de resolución apropiado para estudos poboacionais (é dicir, microsatélites) en *P. calcareum* empregando a tecnoloxía de secuenciación de nova xeración (NGS), co obxecto final de aplicalos a varias poboacións desta especie en fondos de maerl da costa Atlántica Europea.

RESULTADOS E CONCLUSIÓNS

No capítulo 1 desta tese (Pardo *et al.* 2014a), as especies formadoras de maerl do Atlántico Europeo, particularmente da área OSPAR, foron delimitadas utilizando un enfoque taxonómico integrador (Schlick-Steiner et ao. 2010). Desta forma, combinouse información morfolóxica e bioxeográfica con datos de secuencias dos xenomas mitocondrial (a rexión do xene COI-5P xeralmente utilizada como *DNA barcode* en estudos de algas vermellas e doutros grupos taxonómicos) e plastidial (*psb*A, un marcador usado con frecuencia en estudos filoxenéticos dentro da clase Florideophyceae). Ademáis, a información molecular utilizada incluíu secuencias obtidas de materiais tipo de especies formadoras de maerl, como o neotipo

de *Phymatolithon calcareum* e o holotipo de *Mesophyllum sphaericum*. Para delimitar as especies, usouse a ferramenta bioinformática ABGD baseada en distancias, e GMYC como método probabilístico baseado no modelo de coalescencia. Paralelamente, levouse a cabo unha revisión de toda a bibliografía concernente ás especies formadoras de maerl no Atlántico Europeo, que se remonta ata principios do século XX, centrándose tanto nas descricións de especies como na súa distribución. Como resultado deste estudo, foron delimitados 13 taxones con rangos biolóxicos plausibles, a partir dun total de 224 especímenes colleitados dende Svalbard ata as Illas Canarias. Destes taxones, 6 asignáronse a especies xa descritas: *Lithothamion glaciale*, *L. corallioides*, *Phymatolithon calcareum*, *Mesophyllum sphaericum*, *Lithophyllum fasciculatum* e *L. dentatum*. Máis recentemente, *L. fasciculatum* así como coleccións de Irlanda e Bretaña identificadas como *L. dentatum* foron consideradas conespecíficos de *L. incrustans* (Hernández-Kantún et ao. 2015a). Os resultados deste capítulo indican que a maioría das especies que forman maerl teñen pequenas áreas de distribución e obsérvase unha substitución gradual das especies coa latitude. Desta maneira, as latitudes frías do Atlántico Europeo están dominadas por dous membros do xénero *Lithothamnion—L. glaciale* e outra especie pendente de asignar nome (*Lithothamnion* sp.1)—, mentres que L. corallioides e *P. calcareum* son os principais constituíntes dos fondos de maerl na Bretaña francesa e na Canle da Mancha. No Atlántico Ibérico, *P. calcareum* e *L. corallioides* coexisten con outra especie (*Phymatolithon* sp. 3) que chega a ser dominante nos fondos de maerl do sur de Portugal (Carro *et al.* 2014, Pardo *et al.* 2014a). Este estudo supuxo un avance significativo no coñecemento do maerl do Atlántico Europeo, xa que é a primeira vez que un enfoque molecular utilízase para levar a cabo unha avaliación da diversidade específica das súas especies formadoras.

No capítulo 2 abordouse a descrición da nova especie formadora de maerl maioritaria no Atlántico Ibérico, atopada durante o estudo levado a cabo no capítulo 1 (i.e. *Phymatolithon* sp. 3), demostrando que tamén nos fondos de maerl subxace unha forte diversidade críptica (Peña *et al.* 2015b). Finalmente, esta nova especie foi descrita como *Phymatolithon lusitanicum*. Ademáis, no capítulo 2, realizouse un estudo comparativo desta especie con outros membros europeos do xénero *Phymatolithon*. Devandita análise incluíu a obtención de información molecular do material tipo de varias especies, como *Phymatolithon lamii* e *P. laevigatum*. Aínda que *P. lusitanicum* é especialmente abundante en fondos submareales do Atlántico Ibérico, tamén se detectou como maerl no intermareal irlandés e no Mar Mediterráneo Occidental (Mar de Alborán, Illas Baleares) ata os 64 m de profundidade. Ata

agora, *P. lusitanicum* foi atopado unicamente formando maerl. Pola contra, *P. lamii* (unha especie anatomicamente próxima) só se rexistrou baixo forma incrustante, mesmo en mostras recollidas asociadas a fondos de maerl. Dentro deste estudo, tamén descubrimos que a análise molecular do material tipo da especie incrustante *Lithothamnion hamelii* indica a súa conespecificidade con *P. calcareum*.

No capítulo 3 realizouse unha contribución ao coñecemento do ciclo de vida dunha das principais especies formadoras de maerl: *Phymatolithon calcareum* (Peña *et al.* 2014b). Desta maneira, confirmouse con información molecular (usando secuencias do xene COI-5P), a presenza de gametófitos desta especie baixo formas incrustantes. Cabe mencionar que a identificación das mostras de *P. calcareum* foi corroborada utilizando unha secuencia do neotipo desta especie, e tamén con outras secuencias de *P. calcareum* obtidas de especímenes de maerl procedentes de varias rexións do Atlántico Europeo. As mostras incrustantes estudadas procedían dun banco de maerl submareal da Bretaña francesa, curiosamente da mesma zona na que Mendoza & Cabioch (1998) citaron por primeira vez a presenza de gametófitos de *P. calcareum* e *Lithothamion corallioides*, tamén baixo formas incrustantes, usando datos morfolóxicos. Adicionalmente, tamén se realizou un estudo morfolóxico destas coleccións mediante microscopio electrónico de varrido (SEM, "Scanning Electron Microscope"), concluíndo que eran gametófitos femininos a pesar de atopar conceptáculos baleiros. A escaseza de estruturas reprodutoras asexuales no maerl, xunto coa presenza de gametófitos baixo formas costrosas, leva a suxerir que o modo de propagación dominante é vexetativo por simple fragmentación. Hai que destacar que este estudo é o primeiro que confirma a presenza de gametófitos de *P. calcareum* baixo formas incrustantes en base a información molecular.

No último capítulo desta tese (capítulo 4) preséntase o primeiro conxunto de marcadores microsatélite desenvolvidos para un alga vermella coralinácea (Pardo *et al.* 2014b). En concreto, desenvolvéronse oito loci polimórficos (tres dinucleótidos, dous trinucleótidos e tres tetranucleótidos) para *Phymatolithon calcareum*, utilizando a tecnoloxía NGS. Nestes loci detectouse un baixo polimorfismo, pero non parece ser unha característica exclusiva deste tipo de alga coralina, xa que noutras algas vermellas onde se desenvolveron microsatélites, os valores mostrados tamén foron baixos, suxerindo que o baixo polimorfismo parece unha característica típica das algas vermellas en xeral. Por outra banda, os oito loci foron incorporados con éxito nun protocolo de PCR multiplex para facilitar o seu posterior xenotipado. Os microsatélites desenvolvidos proporcionan unha ferramenta adecuada para

realizar estudos sobre a xenética poboacional das especies formadoras de maerl, co fin de incorporar a devandita información aos plans de conservación e xestión desenvolvidos ata agora nestes hábitats de alto valor para a conservación. A este respecto, no Anexo I desta tese (Pardo *et al.* 2015b), móstranse os primeiros resultados obtidos tras a aplicación destes oito microsatélites a varias poboacións de *P. calcareum* en fondos de maerl do Atlántico Europeo, onde se detectou unha forte sinal de clonalidade, aínda que variable, mesmo en poboacións situadas na mesma rexión.

Appropriate management of habitats of conservation concern, such as the maerl beds, requires reliable information on basic issues like species diversity, life cycle, and the population genetics of maerl-forming species. Conventionally, these topics had been addressed with morphological information. In comparison, the present thesis has tried to step forward by using molecular markers to solve these gaps in our knowledge. In chapter 1, maerl-forming species were delimited in Atlantic Europe using an integrative taxonomic approach that included COI-5P and *psb*A sequences as molecular data. A new major maerl-forming species was discovered by DNA barcodes, and its description is provided in chapter 2. A contribution to disentangle the life cycle of a major maerl-forming species (*Phymatolithon calcareum*) was obtained with a combination of DNA barcodes and morphology in chapter 3. Chapter 4 describes the development of a set of microsatellite markers for this species using NGS technology. Finally, preliminary results obtained with these microsatellites to several populations of *P. calcareum* across European Atlantic maerl beds are shown in a final Annex to this thesis.